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Transcriptional profile of primary astrocytes expressing ALS-linked mutant SOD1

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Abstract

Amyotrophic lateral sclerosis (ALS) is caused by the progressive degeneration of motor neurons. Mutations in the Cu/Zn superoxide dismutase (SOD1) are found in about 20% of patients with familial ALS. Mutant SOD1 causes motor neuron death through an acquired toxic property. Although the molecular mechanism underlying this toxic gain-of-function remains unknown, evidence support the role of mutant SOD1 expression in non-neuronal cells in shaping motor neuron degeneration. We have previously found that in contrast to non-transgenic cells, $SOD1^{G93A}$-expressing astrocytes induced apoptosis of co-cultured motor neurons. This prompted us to investigate whether the effect on motor neuron survival was related to a change in the gene expression profile. Through high-density oligonucleotide microarrays, we found changes in the expression of genes involved in transcription, signaling, cell proliferation, extracellular matrix synthesis, response to stress and steroid and lipid metabolism. The most up-regulated gene was decorin (*Dcn*), a small multifunctional extracellular proteoglycan. Down-regulated genes included the insulin-like growth factor-1 receptor (*Igf-1r*) and the RNA binding protein ROD1. *Rod1* was also found downregulated in purified motor neurons expressing $SOD1^{G93A}$. Changes in the expression of *Dcn*, *Igf-1r* and *Rod1* were found in the spinal cord of asymptomatic animals, suggesting these changes occur before overt neuronal degeneration and potentially influence astrocyte-motor neuron interaction in the course of the disease. The astrocyte-specific gene expression profile might contribute to the identification of possible candidates for cell type-specific therapies in ALS.

Keywords

motor neurons; decorin; IGF-1R; ROD1; microarray

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Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease, caused by the progressive degeneration of motor neurons in the spinal cord, brainstem and motor cortex. Motor neuron death leads to muscle weakness and paralysis causing death in one to five years from symptom onset (Rowland & Schneider, 2001). About 10% of ALS shows a familial inheritance and 10–20% of these familial ALS are caused by mutations of the Cu/Zn superoxide dismutase (SOD1) (Rosen *et al*., 1993). A toxic gain-of-function of mutant SOD1 has been considered the cause of neurodegeneration, based on the observation that mice lacking endogenous SOD1 fail to show motor neuron degeneration, while rodents over-expressing human mutant SOD1 generally develop an ALS-like phenotype (Gurney *et al*., 1994; Reaume *et al*., 1996; Howland *et al*., 2002). Several hypotheses, including oxidative stress, glutamate excitotoxicity, formation of high molecular weight aggregates, and mitochondrial dysfunction have been proposed to explain the toxic effect of mutated SOD1 (Beckman *et al*., 2001; Cleveland & Rothstein, 2001; Bruijn *et al*., 2004; Manfredi & Xu, 2005).

Although the molecular mechanism underlying this toxic gain-of-function remains unknown, several lines of evidence suggest that ALS is not a cell-autonomous disease. Transgenic mice expressing mutated forms of SOD1 exclusively in neurons (Pramatarova *et al*., 2001; Lino *et al*., 2002) or astrocytes (Gong *et al*., 2000) fail to develop the disease. Moreover, toxicity to motor neurons requires damage caused by mutant SOD1 expression in non-neuronal cells (Clement *et al*., 2003). A large proportion of non-neuronal cells in the ventral horn of the spinal cord are astrocytes, which closely interact with neurons to provide structural, metabolic and trophic support and actively participate in modulating neuronal excitability and neurotransmission (Volterra & Meldolesi, 2005). Following injury, astrocytes respond by proliferating and adopting a reactive phenotype characterized morphologically by hypertrophic nuclei and cell bodies and by elaboration of distinct long and thick processes with increased content of glial fibrillary acidic protein (GFAP). In addition, morphological alterations are accompanied by changes in the expression of cytoskeleton proteins, cell surface and matrix molecules, proteases, protease inhibitors, several growth factors and cytokines (Ridet *et al*., 1997). Therefore, functional alterations in activated astrocytes can shape the interaction with surrounding cells such as damaged neurons, microglia and immune cells, and consequently could modulate the survival of motor neurons.

Reactive glial changes occur in most neurodegenerative diseases. In ALS patients, a strong glial reaction typically surrounds both upper and lower motor neurons (Hirano 1996; Kushner *et al*., 1991; Nagy *et al*., 1994). Astrocytes in ALS show increased immunoreactivity for GFAP and the calcium binding protein S100β and express inflammatory makers such as COX-2, inducible nitric oxide synthase (NOS) and neuronal NOS (Migheli *et al*., 1999; Sasaki *et al*., 2000; Anneser *et al*., 2001; Maihofner *et al*., 2003). In addition, astrocyte pathology is accompanied by markers of oxidative and nitrative stress (Abe *et al*., 1997; Sasaki *et al*., 2000). A similar pattern of astrocytic changes has been described in mice and rats models of ALS (Gurney *et al*., 1994; Bruijn *et al*., 1997; Hall *et al*., 1998; Almer *et al*., 1999; Nagai *et al*., 2001; Howland *et al*., 2002). This evidence has

prompted us to investigate the role of astrocytes in the disease. Reactive astrocytes in ALS may promote the elimination of damaged motor neurons re-expressing the p75 neurotrophin receptor, while increasing the support for remaining healthy neurons (Pehar *et al*., 2004, Cassina *et al*., 2005; Barbeito *et al*., 2004, Vargas *et al*., 2005). The involvement of these cells in pathogenesis offers a potential target for developing novel strategies to prevent neurodegeneration occurring in ALS.

Primary spinal cord astrocytes monolayers support the survival of highly purified embryonic motor neurons in the absence of any added trophic factor. However, when astrocytes are pre-treated with LPS, peroxynitrite or FGF-1, activated astrocytes cause a 40% neuronal loss over the next 48–72 h (Cassina *et al*., 2002; Pehar *et al*., 2004, Cassina *et al*., 2005). We have reported that in contrast to the trophic support provided by non-transgenic astrocytes, 40% of motor neurons were lost when co-cultured on untreated astrocytes isolated from $SOD1^{G93A}$ rats (Vargas *et al*., 2006). Recently, Nagai et al. (2007) have confirmed our previous results and extended this observation to astrocytes expressing other ALS-linked mutant SOD1. Because the restricted expression of mutant hSOD1 to astrocytes causes the death of non-transgenic motor neurons in our co-culture model, we investigated whether this observation could be related to changes in gene expression induced by $SOD1^{G93A}$ in astrocytes. The differences in gene expression were compared between resting non-transgenic and $SOD1^{G93A}$ spinal cord astrocytes using the Affymetrix microarray system (Rat 230 2.0) to monitor the expression profile of about 28,000 rat genes in astrocytes. The results obtained may help to understand the role of astrocytes and their interaction with motor neurons in ALS.

Materials and Methods

Materials

Culture media and serum were obtained from Gibco-Invitrogen (CA, USA). Primers were obtained from Integrated DNA Technologies, Inc (IA, USA). Anti-Decorin antibody (LF-114) was kindly provided by Dr. Larry W. Fisher (Fisher *et al*., 1995) and anti-IGF-1Rβ was from Santa Cruz Biotech. (CA, USA). Antibodies to GFAP, tubulin and actin were from Sigma (MO, USA). All other reagents were from Sigma unless otherwise specified.

Cell cultures

Primary astrocyte cultures were prepared from spinal cords of 1–2 day old rat pups according to the procedures of Saneto and De Vellis (1987) with minor modifications (Cassina *et al*., 2002). Purified astrocyte monolayers were obtained after 14 days in culture. At this point, astrocytes were plated at a density of 2×10^4 cells/cm^2 and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, HEPES (3.6 g/L), penicillin (100 IU/mL) and streptomycin (100 μg/mL) for additional 7 days. Thus, all experiments were performed with astrocytes cultured for a total of 21 days. The same paradigm was used in our previous co-culture studies where altered astrocyte-motor neuron interaction was observed (Vargas *et al*., 2006). Astrocyte monolayers were >98% pure as determined by GFAP immunoreactivity and devoid of OX42-positive microglial cells. In order to have astrocyte cultures of both genotypes in each preparation, cultures were

prepared from transgenic SOD1^{G93A} and non-transgenic littermates genotyped by PCR. Sprague-Dawley SOD1^{G93A} L26H rats were kindly provided by Dr. David S. Howland (Wyeth Research, Princeton, NJ) (Howland *et al*., 2002). Motor neurons were isolated from embryonic day 15 (E15) rat spinal cord by a combination of metrizamide-gradient centrifugation and immunopanning with the monoclonal antibody IgG192 against p75^{NTR} (Henderson *et al*., 1995).

RNA isolation

Confluent astrocytes monolayers were changed to supplemented L15 medium (Vargas *et al*., 2006) for 24h before RNA isolation. Total RNA was isolated with RNeasy kit/RNase-Free DNase Set (Qiagen, CA, USA). RNA quality was assessed with the $A_{260/280}$ ratio and the 2100 Bioanalyzer (Agilent Technologies, CA, USA) to ensure the integrity of the samples used for microarray analysis.

Animals

Sprague-Dawley SOD1^{G93A} L26H rats were observed daily for onset of disease symptoms as well as progression to death. Onset of motor neuron disease was scored as the first observation of an abnormal gait or evidence of hind limb weakness (typically after 135d). Five asymptomatic (60d) and five at the age of symptoms onset SOD1^{G93A} animals and the same number of age-matched nontransgenic (NonTG) rats were used. Animal procedures followed the guidelines for Care and Use of Laboratory Animals established by the National Institute of Health. Lumbar spinal cords were immediately frozen in liquid nitrogen followed by mortar fragmentation; total RNA was isolated with RNeasy kit (Qiagen) after discarding the upper lipid layer from the lysate in the RLT buffer.

Microarray analysis

Double-stranded cDNA was synthesized from 5 μg of total RNA and used for microarray analysis with the Rat Genome 230 2.0 array (Affymetrix, CA, USA). A total of six arrays divided in three control and three transgenic samples were used. Labeling was performed with one-cycle target labeling assay according to Affymetrix, including the eukaryotic poly-A RNA control and the eukaryotic hybridization control kit. Hybridization, washing and scanning were carried out as described in the Affymetrix GeneChip expression analysis technical manual at the Oregon State University's Center for Genome Research and Biocomputing. Image processing was done using Affymetrix GCOS 1.4 software. The quality of hybridization and overall chip performance was determined by visual inspection of the raw scanned data and the GCOS-generated report file. Microarray data (.CEL files) was normalized using GC-RMA probe-level analysis in ArrayAssist 4.0 (Stratagene, CA, USA). Following variance stabilization and Log transformation, the fold-change versus *p*-value was calculated using nontransgenic samples as base values. Selected probe sets were annotated using NetAffx (www.affymetrix.com/analysis/index.affx) and Gene Ontology Consortium (www.geneontology.org) database in early 2006. Affymetrix probe set identifiers were replaced with official gene nomenclature using NCBI and NetAffx databases. In compliance with MIAME standards, Affymetrix data files (CHP and CEL)

have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository (GSE7441).

Relative quantitative RT-PCR

2 μg of total RNA were randomly reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen, CA, USA) according to the manufacturer's protocol. Specific primers pairs and QuantumRNA Classic 18S Internal Standards primers (Ambion, TX, USA) were used together in the reaction to amplify the target cDNA and a 489 bp from 18S rRNA cDNA. PCR primers were as follows, *Dcn*: 5'-ATCTCCGAAGTTGGGCAGCATG-3', and 5'-CTGAAGGTGTGTGGGTGAATTTGC-3' (125 bp); *Igf-1r*: 5'-CGCAGGATGGCTATCTGTTCCG-3', 5'-GGTCTGGGCACAAAGATGGAGTTG-3' (250 bp); *Rod1*: 5'-ACATCTTCCCTCCGTCAGCCAC-3', 5'-TTGCTTCCTCCACAGATCCCAAC-3' (171 bp). PCRs were carried out in a 50 μl reaction volume containing 1 μl of cDNA, 20 pmoles of each specific primer, 18S primers (1:9 ratio), 200 μM dNTPs, 1.5 mM $MgCl_2$, 1.5 U of *Platinium*Taq and 1X PCR buffer (Invitrogen, CA, USA). The cycling parameters were as follows: 95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec, for 19 to 22 cycles depending on the specific linear range of each amplicon. Minus RT controls were included in each assay. The amplification products were run in non-denaturing 6% polyacrylamide gels and stained with SYBR Gold Nucleic Acid Gel Stain (Molecular Probes, OR, USA). Densitometric analyses were performed using the NIH Image program and gene expression levels were normalized against the 18S levels.

Western blot analysis

Confluent astrocyte culture were washed with cold PBS and whole cell extracts were prepared in 50 mM HEPES, pH 7.5, 50 mM NaCl, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche) and sonicated 3 times for 3 s. Spinal cords were removed and processed in SDS 1% plus Complete protease inhibitor cocktail (Roche) for protein extraction. Protein concentration was measured by the bicinchoninic acid method (Pierce). Protein samples were resolved on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Amersham Biosciences). Membranes were blocked for 1 h in Tris-buffered saline, 0.1% Tween-20, and 5% nonfat dry milk, followed by an overnight incubation with primary antibody diluted in the same buffer. After washing with 0.1% Tween in Tris-buffered saline the membrane was incubated with peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h, and then washed and developed using the ECL chemiluminescent detection system (Amersham Biosciences). Primary antibodies were used at dilutions recommended by the manufacturers.

Immunofluorescence

Astrocyte cultures on Lab-Tek (Nunc, IL, USA) slides were fixed in ice-cold 4% paraformaldehyde in PBS. Briefly, cultures were permeabilized with 0.1% Triton X-100 in PBS for 15 min and blocked for 1h with 10% goat serum, 2% bovine serum albumin, and 0.1% Triton X-100 in PBS. Primary antibodies diluted in blocking solution were incubated overnight at 4 °C. After washing with PBS, fluorophore-conjugated anti-rabbit or anti-

mouse secondary antibodies diluted in blocking solution were incubated for 1h at room temperature. The slides were mounted using ProLong anti-fade kit (Molecular Probes, OR, USA). Primary antibodies used were anti-GFAP polyclonal antibody (1:400) and anti-tubulin monoclonal antibody (1:2000). Secondary antibodies were Alexa Fluor488-conjugated goat anti-rabbit (5 μg/mL; Molecular Probes) and AlexaFluor594-conjugated goat anti-mouse (5 μg/mL; Molecular Probes). Nuclei were stained with DAPI (1 μg/mL; Molecular Probes, OR, USA).

Statistics

Each experiment was repeated at least three times and data are reported as mean ± SD. Comparison of the means was performed by one-way analysis of variance. Pairwise contrasts between means utilized the Student-Newman-Keuls test and differences were declared statistically significant if $p<0.05$. All statistical computations were performed using the SigmaStat Software (Jandel Scientific).

Results

Morphological analysis

Primary spinal cord astrocytes expressing hSOD1^{G93A} are morphologically equivalent to non-transgenic astrocytes in culture, as determined by immunofluorescence for GFAP and α-tubulin (Fig. 1A). SOD1^{G93A} astrocytes did not develop the typical thick and long processes with increased immunoreactivity for GFAP that characterize reactive astrocytes. Accordingly, no increase on GFAP expression was observed by western blot (Fig. 1B). Thus, *in vitro* the expression of mutant SOD1 alone did not cause the typical astrocyte activation found in ALS. However, under the same conditions, SOD1^{G93A} astrocytes induced apoptosis of co-cultured motor neurons (Vargas *et al*., 2006). This prompted us to investigate whether the effect on motor neuron survival was related to changes in the gene expression pattern of astrocytes affecting the interaction with motor neurons.

Gene expression profiling

Microarrays analyses were performed with RNA samples from 3 independent preparations of primary astrocytes obtained from SOD1^{G93A} animals and non-transgenic littermates. We used the Affymetrix Rat Genome 230 2.0 array, which contains over 31,000 probe sets comprising about 28,000 rat genes, followed by GC-RMA normalization. We selected genes with at least 1.2-fold change in non-transgenic and SOD1^{G93A} astrocytes with a statistical significance of $p<0.05$ (Gibson, 2003). The analyses identified a total of 81 transcripts that were statistically different in SOD1^{G93A} astrocytes: 55 transcripts were up-regulated and 26 were down-regulated (Table 1 and 2). The genes were categorized taking into account their molecular function, biological process and cellular component. Approximately half of the genes in each category have no functional information available.

The scatter plot of the fold-change versus the result *p*-value of significance analysis (Fig. 1C), shows that no major changes in the expression pattern were observed in these samples. We applied several probe-level analyses (PLIER, MBEI and MAS5) and obtained similar results, indicating that although these astrocytes displayed a strikingly functional difference,

there is not a prominent difference at the transcriptional level. Interestingly, only the small proteoglycan decorin (*Dcn*) is greatly induced in SOD1^{G93A} astrocytes, whereas only one gene displayed a down-regulation greater than 2-fold in expression (regulator of differentiation, *Rod1*).

Expression of genes previously implicated in ALS

Increased oxidative stress, as a consequence of enhanced reactive oxygen and nitrogen species generation, is known to be involved in ALS. We found increased expression of the stress response DNA-repair protein, aprataxin, which has been associated with increased resistance to oxidative stress (Hirano *et al*., 2006). However, we did not find changes in genes directly associated with increased production of pro-oxidant species or decreased antioxidant defenses. Our study detected no change in the expression of any of the three nitric oxide synthases, although we have previously observed a modest increased accumulation of nitrite and nitrate in the media of transgenic astrocyte-motor neuron co-cultures (Vargas *et al*., 2006).

In addition, no modification in the expression level of the astroglial glutamate transporter EAAT2 were detected, although diminished EAAT2 protein levels reported in ALS could be due to an aberrant splicing of EAAT2 messenger (Lin *et al*., 1998). In contrast to previous reports (Yoshihara *et al*., 2002; Dangond *et al*., 2004), we found an increased expression of GABA-A receptor in SOD1^{G93A} astrocytes. However, the decreased GABA-A receptor expression previously reported might be linked to a late stage of the disease, since this studies were performed in 17-week old ALS-mice and post-mortem human tissue. Within the genes with unknown function, we found increased expression of the gene linked to the oculocerebrorenal syndrome of Lowe. Mutations in this gene have been linked to altered phosphoinositide metabolism and have also been reported to be increased in the spinal cord of sporadic ALS patients (Jiang *et al*., 2005).

Four genes related to cell proliferation were decreased in transgenic astrocytes (Table 2). Among them, the fibroblast growth factor-1, previously reported to be down-regulated in the anterior horn of spinal cord from ALS patients (Kage *et al*., 2001), was found decreased in primary SOD1^{G93A} astrocytes. Our study also detected a decreased expression of IGF-1 receptor gene (*Igf-1r*).

RT-PCR verification

We confirmed the level of expression of *Dcn*, *Rod1* and *Igf-1r* based on the largest fold-change and its putative relevance for in ALS. We used four independent astrocyte samples to assess differential expression by RT-PCR. We confirmed the up-regulation of decorin in SOD1^{G93A} astrocytes at messenger and protein level (Fig. 2A, 2B). We also confirmed the reduced expression of the most down-regulated gene, *Rod1* and the down-regulation of *Igf-1r* obtained from microarray data in SOD1^{G93A} astrocytes (Fig. 2C). IGF-1R protein was also down-regulated in SOD1^{G93A} astrocytes (Fig. 2D).

Expression in spinal cord of SOD1^{G93A} rats and in purified motor neurons

We examined the expression of *Dcn*, *Igf-1r*, and *Rod1* in the spinal cord of SOD1^{G93A} rats (Fig. 3). *Dcn* was found to be up-regulated, while *Igf-1r* and *Rod1* were down-regulated in both asymptomatic (60 days old) and early symptomatic (~135 days old) transgenic animals. The increase in decorin messenger correlated with an increase in protein content in the spinal cord (Fig. 3C), whereas no apparent change in IGF-1R protein was observed (Fig. 3D). When assessed in purified embryonic motor neurons, we observed down-regulation in the expression of *Rod1*, while no changes in the expression of *Dcn* or *Igf-1r* were found (Fig. 4).

Discussion

Although the toxic effect of the gain of function of mutated SOD1 is still under intensive research, the interplay between different cell types has emerged as a major determinant in the pathophysiological mechanisms of ALS (Clement *et al*., 2003, Barbeito *et al*., 2004, Yamanaka *et al*., 2008). In this context, purified primary cultures represent useful tools to identify the primary molecular events of pathological importance. Astrocytes play a pivotal role in nervous system homeostasis and represent a promising target to provide neuroprotection. We have previously reported that the expression of SOD1^{G93A} in astrocytes causes apoptosis in about 40% of co-cultured motor neurons (Vargas *et al*., 2006). However, the present microarray analysis of SOD1^{G93A} astrocytes showed changes in the expression of less than 0.5% of the genes examined even though a low fold change (1.2) cutoff was used. The overall analysis showed a marked degree of transcriptional activation, as has been observed in many genome-wide analyses of tissues from ALS patients or animal models (Malaspina & de Belleroche, 2004). Despite the fact that functional categorization periodically changes and differentially expressed genes are expected to be involved in several pathways, altered expression of genes related to transcription, signaling, cell proliferation, stress responses and steroid and lipid metabolism were observed in SOD1^{G93A} astrocytes. Although we could not directly link genes changes to the toxicity that astrocytes exert towards co-cultured motor neurons, several genes previously implicated in ALS were identified, as well as new candidates that might play a role in the disease.

Rod1 displayed a consistent decreased expression in transgenic astrocytes, motor neurons, asymptomatic and early symptomatic spinal cords. *Rod1* is the functional mammalian homologue of fission yeast's *nrd1*$^{+}$, encoding a RNA binding protein (Yamamoto *et al*., 1999). Although scarce functional data have been reported for ROD1, its overexpression effectively blocks differentiation in human cells without affecting their ability to proliferate. Arrays data also revealed the reduced expression of LIM-domains-containing-1, a gene reported to be down-regulated in human lung cancer and which expression inhibits tumor growth *in vitro* and *in viv*o (Sharp *et al*., 2004). Together with the concomitant reduction in the expression of FGF-1 gene, the results give rise to the possibility that astrocytes in ALS have an altered pattern of proliferation and/or differentiation that may affect its interaction with surrounding cells.

Another interesting implication of *Rod1* down-regulation is its potential participation in RNA processing. *Rod1* is a paralog gene of the polypyrimidine tract binding protein (PTB1)

(Yamamoto *et al*., 1999). PTB1 has diverse roles in pre-mRNA and mRNA function, including the regulation of alternative splicing. Several examples of aberrant RNA splicing exist in neurodegenerative processes (Gallo *et al*., 2005). In particular, the loss of the astroglial glutamate transporter EAAT2 in ALS and the occurrence of an isoform of peripherin that cause motor neuron toxicity has been linked with altered RNA processing (Lin *et al*., 1998; Robertson *et al*., 2003). In this context, the functional consequences of down-regulation of *Rod1* in both astrocytes and motor neurons expressing $SOD1^{G93A}$ mutation deserve further investigation.

Changes in the expression of extracellular matrix-related genes in CNS affect several biological processes including proliferation, neuronal migration and axon guidance. Decorin, a multifunctional proteoglycan controlling matrix construction, was increased more than 4-fold in $SOD1^{G93A}$ astrocytes. Ephrin B1 and Reelin, two genes associated with neuronal migration and axon guidance (Flanagan & Vanderhaeghen, 1998; Jossin *et al*., 2004) were also up-regulated in transgenic astrocytes. Additionally, *Dcn* expression was increased in the spinal cord of $SOD1^{G93A}$ animals. Decorin is up-regulated in astrocytes after CNS injury and attenuates glial scar formation and inflammation (Stichel *et al*., 1995; Logan *et al*., 1999, Davies *et al*., 2004, 2006). These effects are mediated through the capacity of decorin to neutralize all isoforms of transforming growth factor-β (Yamaguchi *et al*., 1990) and modulate epidermal growth factor receptor tyrosine kinase (Santra *et al*., 2002). Thus, up-regulation of decorin may influence matrix remodeling, glial reactivity and inflammation associated to neurodegeneration occurring in ALS. Moreover, decorin has been shown to inhibit neuronal differentiation of adult multipotent neuronal stem/progenitor cells (Barkho et al., 2006). Therefore, the observed up-regulation of decorin may hinder cell replacement therapies in ALS.

Up-regulation of decorin has a potential functional link to the IGF-1R down regulation found in primary astrocytes and spinal cords from $SOD1^{G93A}$ rats. Decorin binds to the IGF-1R (K_d = 18 nM) with an affinity comparable to that of the IGF-1 itself (K_d = 1.2 nM). Decorin binding to IGF-1R causes its activation followed by receptor down-regulation (Schonherr *et al*., 2005). Conversely, increased IGF-1R immunoreactivity and IGF-1 binding sites has been previously observed in the spinal cord of ALS patients and ALS-mice (Doré *et al*., 1996; Chung *et al*., 2003; Wilczak *et al*., 2003). However, other genome-scale analysis have not detected increased expression of *Igf-1r* mRNA in spinal cord of $SOD1^{G93A}$ mice (Olsen *et al*., 2001) or ALS patients (Dangond *et al*., 2004). In addition, we could not observe changes in *Igf-1r* mRNA expression in isolated embryonic motor neurons expressing $SOD1^{G93A}$. The remarkable effect of IGF-1 therapy in prolonging lifespan and in delaying disease onset in $SOD1^{G93A}$ mice (Kaspar *et al*., 2003) contrasts with the limited success of IGF-1 trial in ALS patients (Lai *et al*., 1997; Borasio *et al*., 1998). These results could be consequence of reduced IGF-1 bioavailability due to increased expression of IGF-1 binding proteins in ALS spinal cord (Wilczak *et al*., 2003). Decorin can also bind IGF-1, though with much lower affinity than IGF-1 binding proteins (Schonherr *et al*., 2005). However, in situations in which decorin is strongly over-expressed in a well-delimited region, it may compete for IGF-1 and thus might contribute to the increased IGF-1 binding capacity observed in spinal cord of ALS patients (Doré *et al*., 1996; Wilczak *et al*., 2003). In

this context, increased decorin and decreased IGF-1R expression in astrocytes could have several implications for ALS, because *i)* decorin is able to reduce IGF-1R expression and eventually reduced IGF-1 bioavailability and *ii)* at least some of the beneficial effects of IGF-1 on motor neurons are mediated through astrocytes (Ang *et al*., 1992, 1993).

Primary cultures and transgenic animal models cannot fully recapitulate human pathology. However, candidates genes derived from transcriptional profiles at genome-wide scale can improve our knowledge concerning the pathogenic clues of ALS and allow the identification of targets for cell type-specific therapies. Here we showed that $SOD1^{G93A}$ expression might have functional consequences in RNA processing, in both astrocytes and motor neurons, by down-regulating Rod1. In addition, we showed that $SOD1^{G93A}$ expression in astrocytes causes changes in the expression of Decorin and IGF-1R. These changes were also observed in the spinal cord of $SOD1^{G93A}$ animals but not in cultured motor neurons expressing $SOD1^{G93A}$, suggesting a cell type-specific effect. In particular, Decorin up-regulation might interfere with IGF-1/IGF-1R system by limiting IGF-1 bioavailability and hindering IGF-1R signaling in astrocytes affecting its interaction with surrounding motor neurons and other cell types (Fig. 5).

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The abbreviations used are

ALS	amyotrophic lateral sclerosis
SOD1	Cu/Zn superoxide dismutase
GFAP	glial fibrillary acidic protein
Dcn	decorin
Igf-1r	insulin-like growth factor-1 receptor and Rod1, regulator of differentiation

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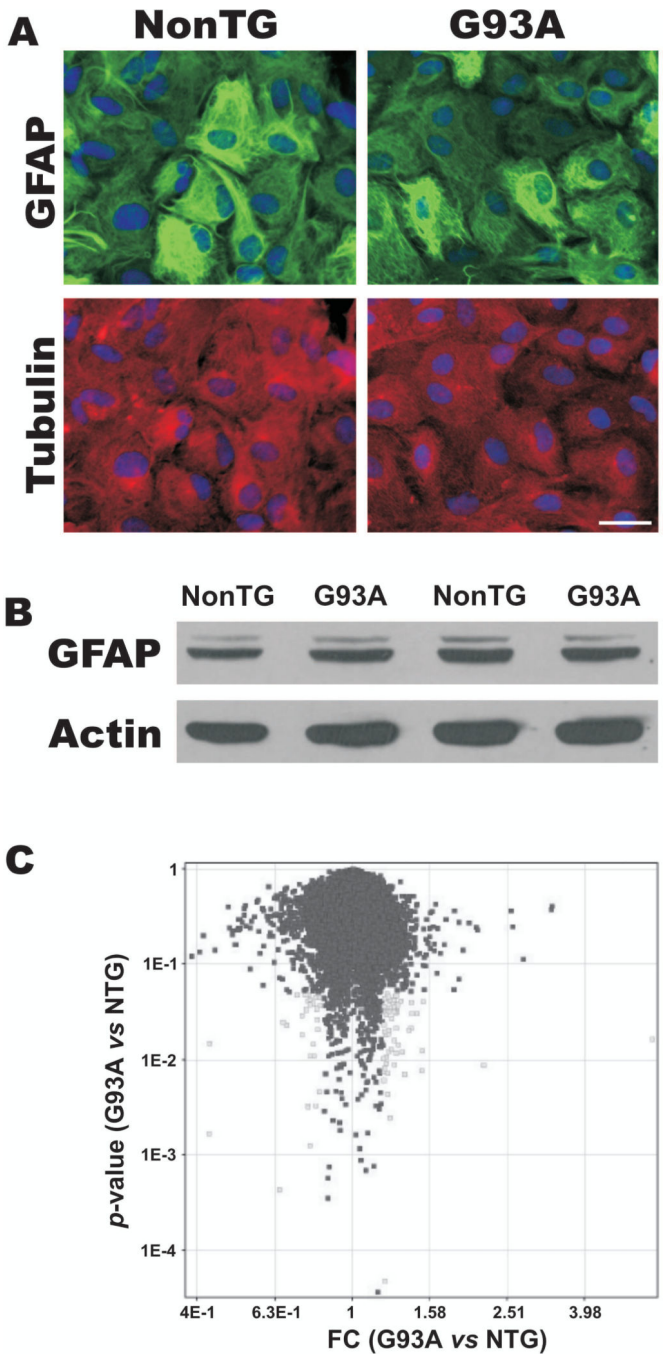

Figure 1.

A) Microphotography showing morphological aspect of non-transgenic (NonTG) and SOD1^{G93A} (G93A) spinal cord astrocyte monolayers. Cultures were stained for GFAP (green) and α-tubulin (red). Nuclei were stained with DAPI. Scale bar: 30 μm. **B)** GFAP protein expression analyzed by western blot in non-transgenic (NonTG) and SOD1^{G93A} spinal cord astrocytes. β-Actin is shown as a loading control. **C)** Scatter plot of the fold-change (FC G93A *vs.* NonTG) versus the result *p*-value of significance analysis. Probe intensity data were normalized using GC-RMA. Following variance stabilization and Log

transformation, the fold-change versus the *p*-value was calculated using non-transgenic samples as base values. Probe sets with significant change ($p<0.05$) of at least 1.2 fold are marked in gray.

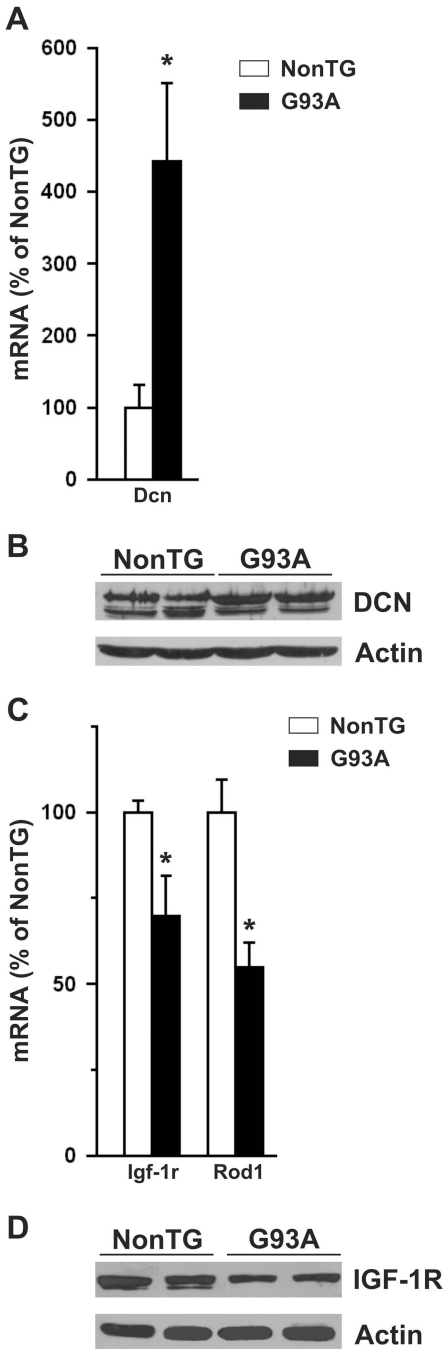

Figure 2.

Verification of selected differentially expressed genes in SOD1^{G93A} astrocytes. Up-regulation of decorin (Dcn) mRNA (**A**) and protein (**B**) was confirmed by relative quantitative reverse transcriptase-PCR and Western blot as described under Materials and Methods. **C)** insulin-like growth factor-1 receptor (Igf-1r) and regulator of differentiation (Rod1) mRNA down-regulation in SOD1^{G93A} astrocytes. **D)** Decreased IGF-1R expression in SOD1^{G93A} astrocytes as determined by Western blot. In (**A**) and (**C**) mRNA levels are

expressed as % of non-transgenic (NonTG) controls and corrected by 18S rRNA cDNA amplification. * Significantly different from control ($p<0.05$).

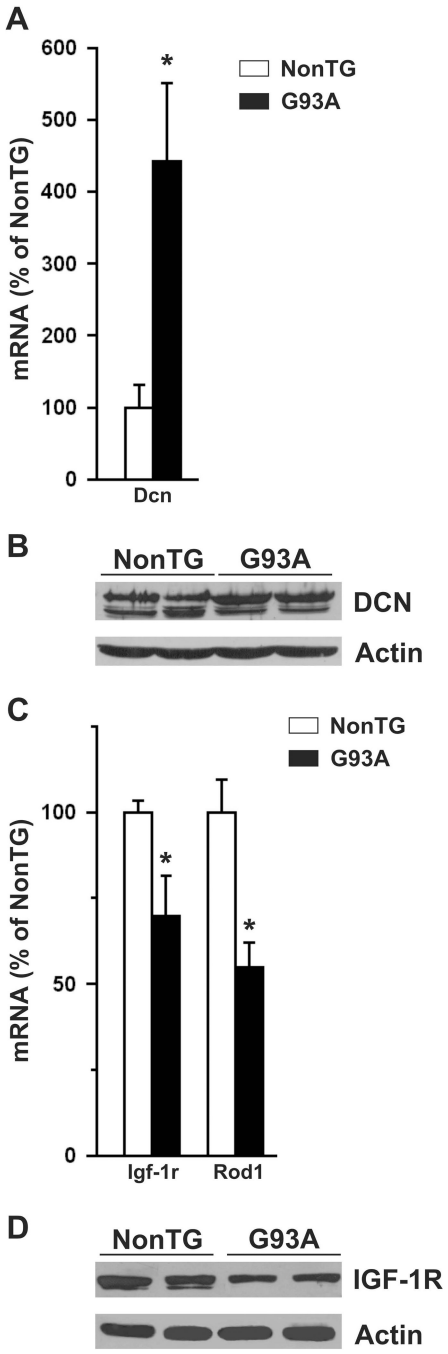

Figure 3.

Expression of decorin (Dcn), insulin-like growth factor-1 receptor (Igf-1r) and regulator of differentiation (Rod1) in the spinal cord of $SOD1^{G93A}$ rats. Comparative mRNA levels from spinal cord of age-matched non-transgenic (NonTG) rats and **A**, asymptomatic or **B**, early symptomatic $SOD1^{G93A}$ rats (G93A). mRNA levels were corrected by 18S rRNA cDNA amplification. Data for each stage were expressed as % of age-matched non-transgenic littermates. * Significantly different from control ($p<0.05$). Decorin (C) and IGF-1R (D) expression levels analysed by Western blot in the spinal cord of asymptomatic (AS) and

early symptomatic (ES) SOD1^{G93A} rats compared to age-matched non-transgenic (NonTG) animals.

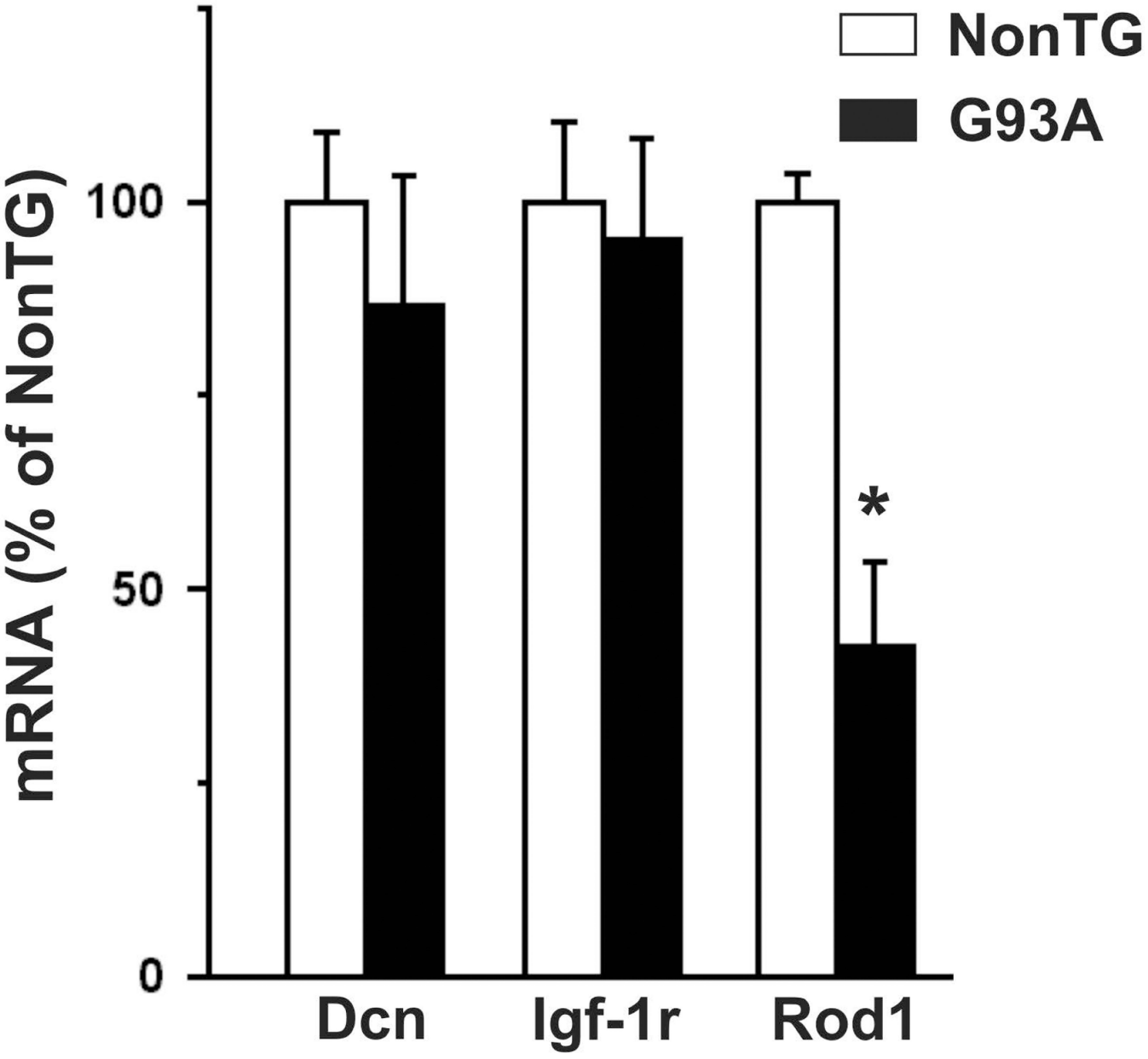

Figure 4.

Expression of decorin (*Dcn*), insulin-like growth factor-1 receptor (*Igf-1r*) and regulator of differentiation (*Rod1*) in isolated spinal cord motor neurons from non-transgenic (NonTG) and $SOD1^{G93A}$-expressing (G93A) E15 embryos. The mRNA levels were corrected by 18S rRNA cDNA amplification and expressed as % of non-transgenic (NonTG) controls. * Significantly different from control ($p<0.05$).

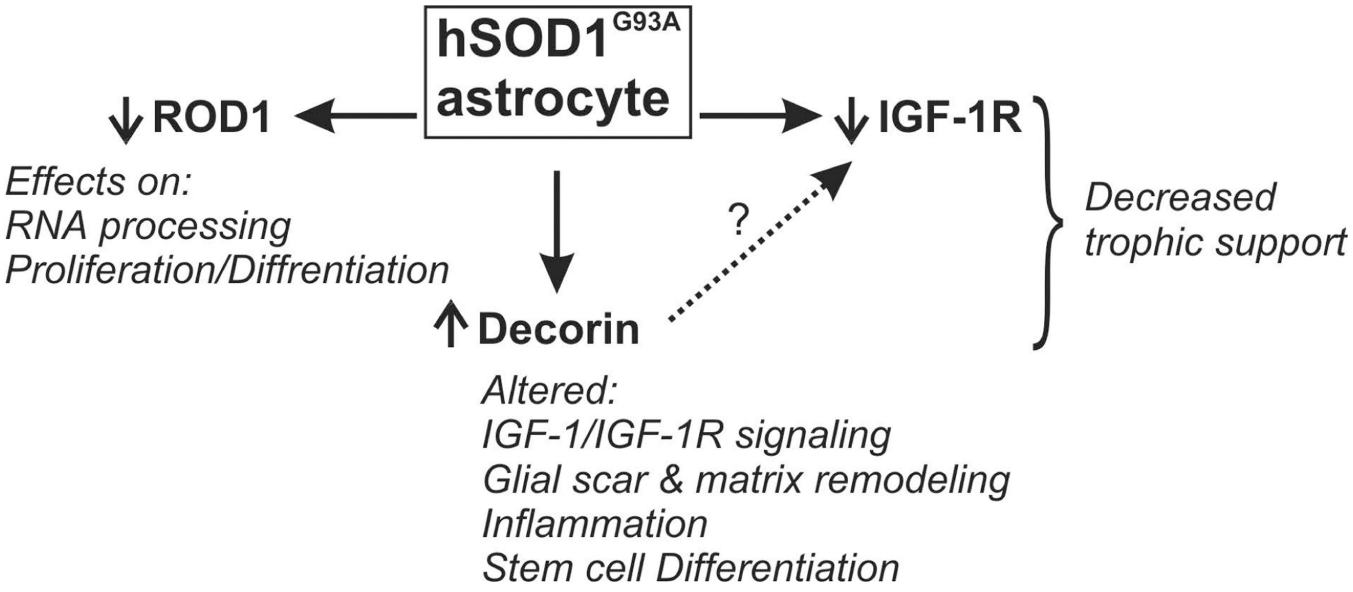

Figure 5.
Schematic representation of some of the changes in gene expression found in SOD1^{G93A}-astrocytes and its putative consequences.

Table I

Genes increased in SOD1^{G93A} astrocytes.

Accession Number	Gene	Fold	p-value
Extracellular matrix/Cell migration			
NM_024129	Decorin	5.95	0.01676
NM_080394	Reelin	1.52	0.04025
NM_017089	Ephrin B1	1.30	0.03252
AI406747	Neuron-glia-CAM-related cell adhesion molecule	1.27	0.03799
Signaling/Receptor activity			
NM_080586	GABA A receptor, gamma 1	1.42	0.03069
AA859669	Neuropilin 2	1.28	0.01612
BC087578	Arrestin, beta 2	1.28	0.00902
NM_053856	Secretogranin III	1.28	0.04208
NM_001012061	Rattus norvegicus Cnksr family member 3 (Cnksr3)	1.25	0.00248
NM_001013192	Similar to olfactomedin-like 1	1.25	0.04174
Transcription			
XM_232488	Atf7 interacting protein (predicted)	1.27	0.03522
NM_057211	Basic transcription element binding protein 1	1.24	0.01243
XM_237754	Zinc finger protein 213 (predicted)	1.23	0.01902
NM_012866	Nuclear transcription factor-Y gamma	1.20	0.00762
Cell Proliferation			
BF408114	Breast cancer metastasis-suppressor 1 (predicted)	1.41	0.02159
Response to stress			
NM_148889	Aprataxin	1.22	0.04865
NM_021863	Heat shock protein 2	1.22	0.01087
Oxidoreductase activity/Metabolism/Transport			
NM_053896	Aldehyde dehydrogenase family 1, subfamily A2	2.18	0.00889
AA997683	Aldehyde dehydrogenase 1 family, member B1 (predicted)	1.47	0.03085
NM_017217	Solute carrier family 7, member 3 (Slc7a3)	1.36	0.00714
XM_224713	Abhydrolase domain containing 8 (predicted)	1.34	0.04100
XM_220931	RAD52 homolog B (S. cerevisiae) (predicted)	1.31	0.03727
L21698	UDP-glucuronosyltransferase 8	1.28	0.04557
AI408770	Villin (predicted)	1.25	0.04092
NM_017268	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	1.21	0.00005
Catalytic activity			
NM_001025017	Similar to 4921507I02Rik protein (predicted)	1.23	0.00441
BI285949	Guanosine monophosphate reductase 2	1.22	0.01854
Unknown function			
BI283971	Transcribed locus	1.51	0.00783
BE103036	cDNA clone	1.51	0.01256
BF400747	Transcribed locus	1.42	0.03988
AW522471	Transcribed locus	1.40	0.02077

Accession Number	Gene	Fold	p-value
BF290416	Transcribed locus	1.34	0.01223
XM_341321	Similar to HCDI protein (predicted)	1.31	0.01455
AI043758	Transcribed locus	1.30	0.02167
AI511280	Similar to DNA segment, Chr 3, ERATO Doi 250, (predicted)	1.30	0.01190
AW528715	Transcribed locus	1.30	0.04675
AA943385	Similar to hypothetical protein FLJ13273 (predicted)	1.29	0.03120
XM_575385	Similar to PHD finger protein 14 isoform 1	1.28	0.03260
BF402699	Transcribed locus	1.26	0.01305
BI276075	Transcribed locus	1.26	0.02746
AI409701	Transcribed locus	1.24	0.04551
AI409069	Similar to MEGF11 protein	1.24	0.02068
BE108716	Similar to MGC68837 protein	1.23	0.03232
XM_343161	MOB1, Mps One Binder kinase activator-like 2B (predicted)	1.23	0.03461
XM_229106	Oculocerebrorenal syndrome of Lowe	1.23	0.00738
BF390720	cDNA clone	1.22	0.00933
AI104921	Transcribed locus	1.22	0.03004
BI303858	Transcription factor Pur-beta	1.21	0.02020
AI101553	Transcribed locus	1.21	0.03558
BI291954	Transcribed locus	1.21	0.04310
AW528635	Transcribed locus	1.21	0.00823
AI070952	Transcribed locus	1.21	0.03260
XM_233227	Similar to KIAA1573 protein (predicted)	1.20	0.00620
XM_227208	Similar to Hypothetical protein MGC57096	1.20	0.01042
NM_001037656	Similar to lactation elevated 1	1.20	0.02391

Table II

Genes decreased in SOD1^{G93A} astrocytes.

Accession Number	Gene	Fold	p-value
Cell proliferation/Signaling			
NM_022282	Discs, large homolog 2 (Drosophila)	−1.52	0.02524
BI289840	Fibroblast growth factor 1	−1.29	0.03758
NM_052807	Insulin-like growth factor 1 receptor	−1.25	0.04427
BI289676	LIM domains containing 1 (predicted)	−1.21	0.03650
Nucleic acid binding			
BE118358	ROD1 regulator of differentiation 1 (S. pombe)	−2.34	0.00167
BE104395	Rearranged L-myc fusion sequence (predicted)	−1.31	0.04095
BC088338	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27 (predicted)	−1.25	0.00335
Oxidoreductase activity/Metabolism			
BF408445	Glutathione peroxidase 7 (predicted)	−1.35	0.01854
NM_012940	Cytochrome P450, family 1, subfamily b, polypeptide 1	−1.35	0.02966
NM_001031662	Coenzyme Q4 homolog (yeast) (predicted)	−1.26	0.01484
Transport			
AI411753	Synaptobrevin-like 1	−1.32	0.03394
X57523	Transporter 1, ATP-binding cassette, (MDR/TAP)	−1.24	0.02683
Actin binding			
XM_341540	Supervillin (predicted)	−1.32	0.04734
Unknown function			
AA957343	Transcribed locus	−1.55	0.00044
XM_217233	Similar to 5-3 exonuclease (predicted)	−1.39	0.04751
BF281893	LOC502928	−1.48	0.02350
AI177465	cDNA clone	−1.31	0.00325
AW532489	Transcribed locus	−1.30	0.00128
XM_574821	Similar to MADS box transcription enhancer factor 2, C	−1.30	0.04758
AI227948	Similar to RIKEN cDNA 1200009B18; EST AA408438	−1.29	0.04216
NM_134419	Hspb associated protein 1 (Hsp27 binding protein)	−1.29	0.01102
BM385061	Transcribed locus	−1.27	0.03868
NM_138975	WW domain binding protein 2	−1.26	0.01114
AI556531	Transcribed locus, strongly similar to NP_060595.2	−1.24	0.03425