INVESTIGATION OF TWO EARLY EVENTS IN AMYOTROPHIC LATERAL SCLEROSIS

-MRNA OXIDATION AND UP-REGULATION OF A NOVEL PROTECTIVE FACTOR MSURI

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder that is characterized by progressive degeneration of motor neurons in the spinal cord, motor cortex and brainstem, which typically results in mortality within 2-5 years after the onset of disease. The cause of disease is unknown in the majority of cases, and there is no cure for ALS of the moment. Approximately 5% of ALS cases are familial, and 15-25% of the familial cases are linked to mutation in the gene encoding the antioxidant enzyme Cu²⁺/Zn²⁺ superoxide dismutase (SOD1). Overexpression of some of ALS-linked mutant SOD1 proteins in transgenic mice results in the development of a neurological disorder that resembles ALS patients. The transgenic mice expressing mutant SOD1 (G93A) is a commonly used ALS animal model. This dissertation demonstrates two early events occurred in the pre-symptomatic stage of SOD1 (G93A) mice, including RNA oxidation and up-regulation of a novel protective factor MSUR1 (mutant SOD1-upregulated RNA 1).

Accumulating evidence indicates that messenger RNA (mRNA) oxidation may be associated with neuronal deterioration during the process of neurodegeneration. The first part of this dissertation is to investigate the relationship between mRNA oxidation and motor neuron degeneration in SOD1 (G93A) mice. It was discovered that mRNA oxidation is an early event far preceding motor neuron degeneration, not merely a
consequence of dying cells, and primarily occurs in motor neurons and oligodendrocytes that die later. Identification of oxidized mRNA species revealed that some mRNA species are more vulnerable to oxidative damage, and importantly, many oxidized mRNA species have been implicated in the pathogenesis of ALS. The expression level of protein corresponding to the oxidized mRNA species is significantly decreased. Furthermore, vitamin E can reduce mRNA oxidation and partially protect motor neurons from neurodegeneration in mice. Increased mRNA oxidation also occurs in the pre-symptomatic stage of other mutant SOD1 mice, including SOD1 (G37R), SOD1 (G85R), and SOD1 (H46R/H48Q) mice. These results suggest that mRNA oxidation may be an important factor initiating the cascade of motor neuron degeneration.

Furthermore, mRNAs are oxidatively damaged in the affected areas of ALS. The phenomena of mRNA oxidation appear to be similar to the symptomatic stage of SOD1 (G93A) mice, suggesting that mRNA oxidation may be a common early event preceding motor neuron degeneration in ALS.

The second part of this dissertation is to investigate a novel transcript named MSUR1, which is upregulated in the pre-symptomatic stage of SOD1 (G93A) mice. MSUR1 is an unusual RNA. It has a typical mRNA structure with a cap at the 5’ end and poly(A) at 3’ end, but is, essentially, a portion of 18S ribosomal RNA (rRNA). It was discovered that MSUR1 is able to rescue SOD1 (G93A)-mediated cell death in HEK293 cells as well as in NSC-34 cells. Expression of MSUR1 significantly reduced SOD1
(G93A)-induced free radical level and oxidative damage. Further, MSUR1 can reduce hydrogen peroxide-mediated cytotoxicity. MSUR1 does not encode a protein, suggesting a functional non-coding RNA. It was widely expressed in various tissues. The results of this study suggest that MSUR1 may be a protective factor which is upregulated against SOD1 (G93A)-mediated cell damage during the pre-symptomatic stage of the disease process in mice.

In conclusion, the results of the first part of this dissertation (mRNA oxidation study) suggest that mRNA oxidation may be a critical factor initiating the cascade of motor neurons degeneration. Antioxidant therapeutic intervention at the earliest possible stage of the disease may be beneficial. The results of the second part of this dissertation (MSUR1 study) suggest that MSUR1 has protective function and may be a potential therapeutic or prevention target for ALS.
Dedicated to my parents and my husband
ACKNOWLEDGMENTS

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PUBLICATIONS

Research Publication


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Major Field: Ohio State Biochemistry Program
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-(OH)dG</td>
<td>8-hydroxy-2'-deoxyguanosine</td>
</tr>
<tr>
<td>8OHG</td>
<td>8-oxo-7' 8-dihydroguanosine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>Aβ</td>
<td>amyloid β peptide</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell leukemia/lymphoma 2</td>
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<td>Cbm</td>
<td>crebellum</td>
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<td>CCS</td>
<td>copper chaperone for SOD1</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>COX</td>
<td>cytochrome c oxidase</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>Cu</td>
<td>copper</td>
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<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
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<td>EAAT2</td>
<td>excitatory amino acid transporter 2</td>
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<td>EAAT3</td>
<td>excitatory amino acid transporter 3</td>
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<td>EGFP</td>
<td>enhanced green fluorescence protein</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FALS</td>
<td>familial amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FC</td>
<td>frontal cortex</td>
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<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<td>GFAP</td>
<td>glial fibrillary acid protein</td>
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<td>H2O2</td>
<td>hydrogen peroxide</td>
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<td>HD</td>
<td>Huntington's disease</td>
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<tr>
<td>hr</td>
<td>hours</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>hSOD1</td>
<td>human Cu/Zn superoxide dismutase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>KD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
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<tr>
<td>MAP2</td>
<td>microtubule associated protein 2</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MCI</td>
<td>mild cognitive impairment</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>MT</td>
<td>Metallothionein</td>
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<td>MUSR1</td>
<td>mutant SOD1-upregulated RNA 1</td>
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<tr>
<td>NF-H</td>
<td>neurofilament heavy chain</td>
</tr>
<tr>
<td>NF-L</td>
<td>neurofilament light chain</td>
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<tr>
<td>NF-M</td>
<td>neurofilament medium chain</td>
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<tr>
<td>NFT</td>
<td>neurofibrillary tangles</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O2(^{\cdot-})</td>
<td>superoxide radical</td>
</tr>
<tr>
<td>OH(^{\cdot})</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>ONOO(^{-})</td>
<td>peroxynitrite</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>pAb</td>
<td>polyclonal antibody</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phospate buffer saline</td>
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<td>PCM-1</td>
<td>pericentriolar material 1</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>proteolipid protein</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>SALS</td>
<td>sporadic amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslational region</td>
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</table>
CHAPTER 1

INTRODUCTION

1.1 Amyotrophic lateral sclerosis

Amyotrophic Lateral Sclerosis (ALS), also called Lou Gehrig's disease in the United States, was first identified in 1869 by the French neurologist, Jean-Martin Charcot. It is a late-onset, devastating fatal neurodegenerative disorder that is characterized by progressive degeneration of motor neurons in the spinal cord, motor cortex and brainstem results in muscles weakness, atrophy, and spasticity (D. W. Mulder et al., 1986; M. Sinaki and D. W. Mulder, 1986). ALS most commonly affects people between 40 and 60 years of age, and men are affected slightly more often than women (1.4:1). The incidence of ALS is 1-2 per 100,000, while prevalence is 4-6 per 100,000 of total population. Early symptoms of ALS include weakness in the extremities, dysphagia, dysarthria and head drop. About 75% of the patients show onset in the limbs, while 20% present with onset in the bulbar area. In later stages of the disease, individuals have difficulty breathing, and eventually die from respiratory system failure, due to denervation of the respiratory muscles and diaphragm. Approximately 5%-10% of ALS cases follow a familial inheritance (Rosen et al., 1993).

Most cases (90%-95%) of ALS have no inherited links, and are thus termed sporadic ALS (SALS). Both familial ALS (FALS) and sporadic ALS produce
similar pathological changes and symptoms. There is no single specific test for the diagnosis of ALS. The diagnosis is usually made by physical examination, electrophysiological findings and by process of elimination of other diagnostic possibilities.

<table>
<thead>
<tr>
<th>Familial ALS/MND loci</th>
<th>Gene</th>
<th>Chromosomal location</th>
<th>References</th>
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<tr>
<td>Adult onset dominant typical ALS</td>
<td>SOD1</td>
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<td>ALS3</td>
<td>16q12</td>
<td>Hand et al., 2002, Abalkhail et al., 2003</td>
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<td>Chance et al., 1998</td>
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<td>ALS2</td>
<td>ALS5</td>
<td>15q15.1–q21.1</td>
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Table 1.1 Genetics of Familial ALS or Other ALS-like Motor Neuron Diseases

The prognosis of ALS is poor, as progressive deterioration of motor neurons results in death within 2-5 years after diagnosis (Bruijn et al., 2004). There is no cure for
ALS. Riluzole, a glutamate antagonist, is the only pharmacologic treatment for ALS that has been approved by the United States Food and Drug Administration (FDA).

It has been reported that riluzole by reducing glutamate excitotoxicity, could prolong life expectancy by 2 months on average (Bensimon et al., 1994). Researchers have identified eight FALS loci, and six related genes, in familial ALS-like motor neuron diseases through genetic linkage studies, defined as ALS1 through ALS8, which have each been associated with ALS alone, as well as ALS with Parkinsonism dementia, and ALS with frontotemporal dementia (Table 1.1).

1.1.2 Superoxide dismutase 1 (SOD1)

Superoxide dismutase 1 (SOD1) is the most common one among many identified causative genes, and mutations in SOD1 account for 15-25% of the cases of familial ALS (FALS). The normal function of SOD1 is to catalyze the reduction of superoxide anion ($O_2^-$) to molecular oxygen $O_2$ and hydrogen peroxide ($H_2O_2$). As such, it is an important antioxidant defense in all cells exposed to oxygen. It is ubiquitously expressed in the cytosol of all cell types, and constitutes approximately 1% of the soluble protein in human brain. The human SOD1 gene has five exons and four introns, and spans 12 kilobases on chromosome 21, where 117 disease-associated mutations have been identified. The human enzyme is a 32-kDa homodimer, with one copper- and one zinc-binding site per 153-amino acid subunit, with the copper binding site is the active site (Fig. 1.1). The finding of essentially normal SOD activity for some mutants (D. R. Borchelt et al., 1994), combined with the fact that SOD1 knock out mice does not result in motor neuron disease, led to the conclusion that the mutant SOD1 causes ALS by acquiring a novel toxic function (Shefner et al., 1999).
Figure 1.1 Crystal structure of human SOD1. Copper and zinc ions are shown as blue and orange spheres, respectively (Valentine et al., 2005). (The figure was adapted from Valentine et al., 2005 annu. rev.biochem Vol. 74: 563-593)
1.1.3 Transgenic mouse models of ALS

Transgenic mice overexpressing the mutated human SOD1 gene develop phenotype and clinical symptoms resembling human ALS (M. E. Gurney, 1994; D. W. Cleveland and J. D. Rothstein, 2001). Partial common mutant SOD1 transgenic mouse models used in ALS studies were listed in Table 1.2.

Mutant human SOD1(G85R), SOD1(G37R), and SOD1(G93A) transgenic mouse models have been commonly used in the study of pathogenesis of ALS (M. E. Gurney, 1994; P. C. Wong et al., 1995; L. I. Bruijn and D. W. Cleveland, 1996). Different SOD1 mutants have remarkable variations of superoxide scavenging activity and protein stability.

Mice overexpressing mutant human SOD1 (G93A) or (G37R) display vacuolar pathology changes (M. E. Gurney, 1994; P. C. Wong et al., 1995), but these are not found in other mutant SOD1 transgenic mice. It is believed that these vacuolizations are due to both G37R and G93A containing full superoxide scavenging activity.

In G85R (line 148) mice, mutant SOD1 protein levels are quite low, and equal to that of endogenous mouse SOD1. This mimics the situation in human ALS (Williamson et al., 1998). Heterozygous SOD1 (G85R) mice have a similar age of disease onset to SOD1 (G37R) mice (approximately 12 months of age).

In heterozygous SOD1 (G85R) mice, slow axonal transport inhibition is the earliest known abnormality; preceding any other pathological changes (Williamson and Cleveland, 1999).

G127X is another mutant discovered from some familial ALS patients. A truncated protein with five novel amino acids following Gly127 was generated by
mutant G127X (Jonsson et al., 2004). Both G85R and the truncated G127X mutant SOD1 are unstable, lacked enzyme activity and had no disulphide bond. G127X also has low protein level of SOD1.

H46R/H48Q are mutations at two of the four histidine residues that are essential for the coordinated binding of copper. H46R/H48Q possesses little or no SOD1 activity.
<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>hSOD1m RNA level /mSOD1</th>
<th>hSOD1 protein level /mSOD1</th>
<th>Life span (day)</th>
<th>hSOD1 activity/stability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>G93A</td>
<td>40</td>
<td>17</td>
<td>124</td>
<td>Y/S</td>
<td>Jonsson et al 2005</td>
</tr>
<tr>
<td>G93A dl</td>
<td>20</td>
<td>8</td>
<td>253</td>
<td>Y/S</td>
<td>Jonsson et al 2005</td>
</tr>
<tr>
<td>G37R29</td>
<td>--</td>
<td>5</td>
<td>365</td>
<td>Y/S</td>
<td>Wong et al 1994</td>
</tr>
<tr>
<td>G37R42</td>
<td>--</td>
<td>12</td>
<td>154</td>
<td>Y/S</td>
<td>Wong et al 1994</td>
</tr>
<tr>
<td>G85R</td>
<td>17</td>
<td>0.9</td>
<td>345</td>
<td>N/U</td>
<td>Brujin et al 1997, Jonsson et al 2004</td>
</tr>
<tr>
<td>G127X</td>
<td>25</td>
<td>0.45</td>
<td>250</td>
<td>N/U</td>
<td>Jonsson et al 2004</td>
</tr>
<tr>
<td>H46R/H48Q</td>
<td>--</td>
<td>--</td>
<td>365</td>
<td>N/S</td>
<td>Wang et al 2002</td>
</tr>
</tbody>
</table>

**Table 1.2** Summary of common used ALS mutant SOD1 transgenic mouse models

Note: Y means yes, N means no, S means stable, and U means unstable.
<table>
<thead>
<tr>
<th>Therapeutic interventions</th>
<th>Mouse strain</th>
<th>Prolonged lifespan</th>
<th>Note</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pharmacological treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine (diet)</td>
<td>G93A</td>
<td>20 days</td>
<td>energy supply</td>
<td>Klivenyi et al, 1999</td>
</tr>
<tr>
<td>Riluzole (diet)</td>
<td>G93A</td>
<td>10-15 days</td>
<td>anti-glutamatergic</td>
<td>Gurney et al, 1998</td>
</tr>
<tr>
<td>Vitamin E (diet)</td>
<td>G93A</td>
<td>no effect</td>
<td>anti-oxidant</td>
<td>Gurney et al, 1996</td>
</tr>
<tr>
<td>Gabapentin (diet)</td>
<td>G93A</td>
<td>no effect</td>
<td>mimic the structure of GABA</td>
<td>Gurney et al, 1996</td>
</tr>
<tr>
<td>Lysine Acetyl-salicylate (diet)</td>
<td>G93A</td>
<td>no effect</td>
<td>a soluble salt of aspirin</td>
<td>Barneoud et al, 1999</td>
</tr>
<tr>
<td>Minocycline (diet)</td>
<td>G37R</td>
<td>21-35 days</td>
<td>tetracycline antibiotics</td>
<td>Kriz et al, 2002;</td>
</tr>
<tr>
<td></td>
<td>G93A</td>
<td>11-21 days</td>
<td>tetracycline antibiotics</td>
<td>Van den et al, 2002</td>
</tr>
<tr>
<td>Cocktail of minocycline-riluzole-nimodipine</td>
<td>G37R</td>
<td>42 days</td>
<td>inhibit microglia activation; anti-apoptosis</td>
<td>Kriz et al, 2003</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>G93A</td>
<td>10 days</td>
<td>cephalosporins</td>
<td>Rothstein, et al, 2005</td>
</tr>
<tr>
<td>Ginseng</td>
<td>G93A</td>
<td>7 days</td>
<td>derived from plant roots, &quot;adaptogens&quot;</td>
<td>Jiang et al, 2000</td>
</tr>
<tr>
<td>zVAD-fmK (i.c.v.)</td>
<td>G93A</td>
<td>27 days</td>
<td>broad caspase inhibitors, toxic for human WT hSOD1 intraspinal infusion</td>
<td>Li et al, 2000</td>
</tr>
<tr>
<td>SOD1 injection</td>
<td>G93A</td>
<td>24 days</td>
<td></td>
<td>Turner et al, 2005</td>
</tr>
<tr>
<td>AOL 10150</td>
<td>G93A</td>
<td>20 days</td>
<td>antioxidant</td>
<td>Crow et al, 2005</td>
</tr>
<tr>
<td><strong>Viral gene therapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF1</td>
<td>G93A</td>
<td>37 days</td>
<td>retrograde viral delivery</td>
<td>Kaspar et al, 2003</td>
</tr>
<tr>
<td>GDNF</td>
<td>G93A</td>
<td>25 days</td>
<td>retrograde viral delivery</td>
<td>Wang et al, 2002</td>
</tr>
<tr>
<td>VEGF</td>
<td>G93A</td>
<td>40 days</td>
<td>retrograde viral delivery</td>
<td>Azzouz et al, 2002</td>
</tr>
<tr>
<td>Cardiotrophin</td>
<td>G93A</td>
<td>27 days</td>
<td>adenovirus gene transfer</td>
<td>Bordet et al, 2001</td>
</tr>
<tr>
<td>RNAi</td>
<td>G93A</td>
<td>100 days</td>
<td>retrograde viral delivery</td>
<td>Ralph et al, 2005; Raoul et al, 2005;</td>
</tr>
</tbody>
</table>

**Table 1.3** Effect of therapeutic interventions on mutant SOD1 transgenic mouse models.
The causes for ALS are unknown; the end-stage ALS patient post mortem tissues are a very limited resource for scientists to unravel the mechanisms involved in neurodegeneration in ALS. Fortunately transgenic mice provide us a way to better understand the pathogenic pathways of ALS and to test different therapeutic approaches. Table 1.2 lists a summary of therapeutic interventions have been tested in ALS transgenic mouse models.

1.1.4 Non-Cell-Autonomous Death of motor neurons

More and more evidence shows that mutant SOD1-mediated cell death is non-cell autonomous; that is, mutant SOD1-mediated damage is required within both motor neurons and non-neuronal cells in order to develop the disease. Mutant SOD1 was expressed selectively in astrocytes (Gong et al., 2000) or in neurons (A. Pramatarova et al., 2001; M. M. Lino et al., 2002) is insufficient to cause the disease, however, the mutant SOD1 level within the neurons maybe too low to yield the disease. Clement et al. (2003) generated the chimeric mice that were mixtures of mutant SOD1-expressing cells and normal cells. They found that some mice with only a small portion of normal cells escaped the disease, despite the high percentage of the mutant-expressing motor neurons. Boillee et al. 2006 demonstrate the contribution of motor neurons and nonneuronal neighbors in triggering the disease; they reported that excision of the “floxed” mutant SOD1 gene within motor neurons can extend survival by slowing disease onset (Boillee et al., 2006). On the other hand, diminishing mutant SOD1 levels within microglia and peripheral macrophages can slow the disease progression at later disease stages and can extend survival significantly but not the disease onset. A similar study, carried out by Beers et al. (2006) demonstrated that transplantation of normal bone marrow
cells into myeloid synthesis defective (PU1 knockout) mutant SOD1 mice had no effect on disease onset, but slowed disease progression. And transplantation of mutant SOD1 myeloid cells (microglia) into normal PU.1<sup>-/-</sup> mice failed to induce ALS-like disease (D. R. Beers et al., 2006). These results indicate that mutant SOD1 within microglia cells can accelerate the disease progression; however, expression of mutant SOD1 in microglia is not sufficient to cause motor neuron death. It also suggests that initial damage to motor neurons is further exacerbated by the surrounding damaged astrocytes and microglia, resulting in further disease progression. The non-autonomous cell death theory was further supported by two recent in vitro studies using embryonic stem cells. They demonstrate that astrocytes play a critical role in the survival of motor neurons; indeed the soluble factors secreted from mutant astrocytes are able to kill the motor neurons in a bax-dependant cell death pathway (F. P. Di Giorgio et al., 2007; M. Nagai et al., 2007). Moreover, Francesco and colleagues observed some cell autonomous degeneration of the embryonic stem cells derived motor neurons. Their results are exciting; however, are contrary to the in vivo study, where expression of mutant SOD1 within astrocytes only in mice does not yield disease (Di Giorgio et al., 2007).

The final downstream event of mutant SOD1-mediated ALS is activation of a cascade of caspase-triggered motor neuron death. Caspase 3 activation in glia and motor neurons is the last step of motor neuron death in mutant SOD1-mediated familial ALS (Friedlander, 2003).

1.1.5 Mitochondrial dysfunction and ALS

Mitochondria, as the power sources of cells, play a critical role in several metabolic processes and apoptotic pathways. Increasing evidence suggests
that mitochondrial dysfunction may be involved in the pathogenesis of ALS. Both morphological and functional abnormalities in mitochondria have been demonstrated in ALS patients and ALS transgenic mouse models. Mitochondrial morphological and ultrastructual abnormality were observed in human postmortem and biopsy samples including skeletal muscle, intramuscular nerves, proximal axons and spinal cord (anterior horn region) of SALS patients. Consistent with these findings, the presence of vacuolated mitochondrial remnants within spinal motor neurons was observed at very early stage in transgenic mice overexpressing mutant SOD1 (G93A) and SOD1 (G37 R). However, these morphological changes are present in SOD1 (WT) transgenic mice, which do not develop disease and are not found in some other transgenic ALS models. For example, SOD1 (G85R) mice do not show any mitochondrial morphological defects. In addition, there are numerous studies demonstrate that mitochondria are functionally abnormal in ALS. Deficits in the activities of mitochondrial respiratory chain complex I and complex IV have been identified in SALS spinal cords (F. R. Wiedemann et al., 1998; G. M. Borthwick et al., 1999). Moreover, a significant loss of mitochondrial membrane potential, a decrease in ATP production levels, impaired electron transport chain activities, and increased ROS production have been reported in a motor neuron-like cells and neuronal cell lines expressing mutant SOD1. These enzymatic deficits may be attributable to mutant SOD1 being trapped in the mitochondrial intermembrane space.

The presence of mutant SOD1 instead of wild- type SOD1 within mitochondria of spinal cord in mutant SOD1 (G93A, G37R, G85R, and G127X) transgenic mice, has been described by several groups. There are similar findings from ALS patient post-mortem tissues. The recruitment of mutant SOD1 into the mitochondria was
observed in spinal cord tissue, but was not seen in unaffected areas. The trapped SOD1 has been found in the intermembrane space, matrix, and both the inner and outer membranes in both the spinal cord and brain mitochondria. How mutant SOD1 impairs mitochondria function is unknown. Liu, et al. demonstrates that mutant SOD1 preferentially associated with mitochondrial, causing clogging of the import machinery, which affects mitochondria normal function (J. Liu et al., 2004). Pasnelli and colleagues reports that mutant SOD1 trapped into mitochondria interacts with Bcl-2, an anti-apoptotic protein, reducing the motor neurons protection from apoptotic mechanisms, thus providing a clue as to of how the entrapped SOD1 may incur harm to the motor neurons (P. Pasinelli et al., 2004). This is supported by studies showing that genetically increasing Bcl-2 expression can delay the disease onset (Vukosavic et al., 1999). The critical question remains unanswered: what are the unique characteristics of mitochondria within the spinal cord that causes the mis-location of mutant SOD1, or are there any special chaperones within the spinal cord facilitate the mis-location of mutant SOD1?

Moreover, mis-targeting mutant SOD1 to the mitochondria in cultured cells is toxic (H. Takeuchi et al., 2002). A mitochondrial donation study carried out by Swerdlow and his colleagues show that the cytoplasmic hybrid cells, generated by fusing ALS patient’s platelets with the neuroblastoma cells depleted of mitochondrial DNA, have impaired respiratory chain function, and increased ROS production (R. H. Swerdlow et al., 1998). Gajewski and his colleges failed to replicate this impairment, when fusing ALS’s platelets with other non-neuronal cell types (Gajewski et al., 2003). Another piece of evidence that mitochondrial metabolism is affected is supported by the fact that administration of creatine, molecules involved in enhancing energy storage
capacity, prolongs the survival in SOD1 (G93A) transgenic mice (P. Klivenyi et al., 1999). These studies did provide evidence of mitochondrial involvement in ALS pathogenesis; however, they did not demonstrate clearly whether mitochondria dysfunction is a primary toxicity of mutant SOD1 or whether it is a secondary byproduct of neurodegeneration.

Mitochondria are not only the energy source of cells, but also the gatekeepers of cell death. They are able to initiate an apoptosis pathway by opening the permeability transition pore, in turn releasing cytochrome c into the cytosol. In the cytosol, cytochrome c interacts with other molecules, activating the caspase cascade to initiate cell death. Although mechanisms leading to motor neuron death in ALS are still unknown, numerous studies indicate that the mitochondrial apoptotic pathway plays a role (Friedlander, 2003). It has been reported that the executioner proteases are activated in ALS, and inhibition of these caspases either genetically or pharmacologically, is beneficial, postponing the disease onset and prolong the lifespan (M. Li et al., 2000). Administration of minocycline, a molecule is thought to inhibit microglia activation and release of cytochrome c from mitochondria, is able to slow the disease progression up to four weeks in mice expressing SOD1 (G93A) and SOD1 (G37R), thus providing evidence that mitochondria-dependent apoptosis is a predominant pathway initiating cell death in ALS (J. Kriz et al., 2002; L. Van Den Bosch et al., 2002).

1.1.6 Protein aggregates and ALS

Protein aggregates or inclusions are a common cytopathological hallmark of many neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, ALS, Huntington’s disease, and Prion diseases, etc. However, whether protein aggregates
or inclusions are, themselves, neurotoxic is unknown.

Cytoplasmic protein aggregates within motor neurons and some surrounding astrocytes have been observed in sporadic ALS, familial ALS patients, and ALS transgenic mouse models as well (M. E. Gurney, 1994; P. C. Wong et al., 1995; L. I. Bruijn et al., 1997b; L. I. Bruijn et al., 1998). In some mutant SOD1 transgenic mouse models, detergent-insoluble form of SOD1 was found within these inclusions. These detergent-insoluble proteins were only detected in the disease affected area (J. A. Johnston et al., 2000; H. X. Deng et al., 2006; Y. Furukawa et al., 2006).

The hypothesized mechanism of protein aggregates toxicity hypothesis includes: (1) loss of protein function due to aggregation, (2) dysfunction of protein degradation (ubiquitin-proteasome), (3) insufficiency of protein folding chaperone machinery.

Ubiquitin has been found in the aggregates in both SOD1-mediated familial ALS patients and in all ALS transgenic mice (P. G. Ince et al., 1998; M. Watanabe et al., 2001; J. Wang et al., 2003). The major function of ubiquitin is targeting proteins for disposal via proteasome machinery. In non-neuronal cells expressing mutant SOD1, protein aggregates were observed followed by proteasome inhibition. Proteasome dysfunction has been reported in ALS (E. Kabashi et al., 2004; K. Puttaparthi and J. L. Elliott, 2005). However, whether proteasome dysfunction is the primary cause or simply the consequence of protein aggregation remains to be elucidated.

In addition to dysfunction of proteasome degradation machinery, an overall reduction in protein-folding chaperone activity, (e.g. Heat- shock proteins (Hsp)), was observed. Shinder et al. (2001) reported a direct interaction of HSP70, HSP40, and αβ-crystallin with mutant SOD1, suggesting involvement of these components (Shinder
et al., 2001). More importantly, expression of several heat shock proteins (Hsp27, Hsp 40, and Hsp70) in cultured primary motor neurons expressing G93A SOD-1 can reduce formation of mutant SOD-containing protein aggregates and prolonged cell survival (W. Bruening et al., 1999; H. Takeuchi et al., 2002; Y. J. Patel et al., 2005). However, overexpressing heat shock protein 70 in ALS mutant SOD1 transgenic mice are insufficient to affect the disease duration or ameliorate pathology (J. Liu et al., 2005).

### 1.1.7 Deficits in axonal transport and ALS

Motor neurons are extremely large, polarized cells with axons extending long distances from the cell bodies. The cell bodies of motor neurons are the center of metabolism, and must continuously transport the newly synthesized protein or energy to axons; on the other hand, proteins in the axon that need to be degraded, must be in the transported back to the cell bodies. Axonal transport is achieved by the interaction of transport proteins with cytoskeletal proteins. There are three kinds of neuronal cytoskeleton components, including actin microfilaments, microtubules and intermediate filaments.

Axonal transport defects have been reported in mutant SOD1 transgenic mouse models. Reduced slowly, anterograde transport was observed at a pre-symptomatic stage (B. Zhang et al., 1997; T. L. Williamson and D. W. Cleveland, 1999). Further, not only the anterograde transports, but also the retrograde transports were affected. Cytoplasmic dynein and dynactin complex, the motors driving retrograde transport in neurons were inhibited in a transgenic mouse overexpressing mutant SOD1. In addition, dynein was found to be co-localized with the mutant SOD1 aggregates in motor neurons (Ligon et al., 2005). Surprisingly, when the SOD1 (G93A) transgenic mouse was crossed to a
dynein point mutation transgenic mouse, the axonal transport defects were rescued, and the life span of the double transgenic mice was prolonged, as compared to the single-transgenic SOD1 mutant mice (Kieran et al., 2005). The mechanism of this rescue is not clear.

Moreover, mutation of DCTN1 gene which encodes for p150\textsuperscript{Glued}, the largest subunit of dynactin, was identified in several families that developed slowly progressive motor neuron disease (I. Puls et al., 2003). Normally functioning of p150\textsuperscript{Glued} serves as a bridge between cytoplasmic dynein and the microtubules, and is essential for coordinated bidirectional motility (K. T. Vaughan and R. B. Valle, 1995; C. M. Waterman-Storer et al., 1995; M. Haghnia et al., 2007). Overexpression dynamitin can disassemble the dynactin complex. Mice overexpressing dynamitin which can disassemble dynactin developed a late-onset progressive motor neuron degenerative disease (B. H. LaMonte et al., 2002). This evidence suggests that axonal defects may be a susceptible factor for the pathogenesis of ALS.

1.1.8 Neurofilaments and ALS

The genes encoding three neurofilament subunits have long been suspected as potential causative factors for ALS because of their link with motor neuron pathology in mice and humans. Aberrant neurofilament accumulations are a pathological hallmark of both familial and sporadic ALS (D. G. Munoz et al., 1988; H. Mizusawa et al., 1989). The accumulations of neurofilaments may enhance the axonal transport defects. Mice with increased expression levels of wild-type NF-H or NF-L subunits develop age-dependent motor neuron pathology (F. Cote et al., 1993; Z. Xu et al., 1993), while expression of a point mutation in NF-L produces fatal, progressive paralysis (Lee et al., 1994).
Deletion of NF-L prolongs the life span in ALS transgenic mice (SOD1 G85R and SOD1 G37R) (T. L. Williamson et al., 1998; M. D. Nguyen et al., 2001). Taken together, abnormal organization to neurofilaments is considered as a potential risk factor for ALS.

1.1.9 Oxidative stress and ALS

Oxidative stress is considered one of the suspects in mutant SOD1-mediated disease. Potential ROS as by-products of aerobic metabolism may arise from mitochondria, xanthine oxidase; cytochrome P450-based enzymes, and NADPH oxidases. Since mitochondria consume about 85-90% of cellular oxygen, they are the major site of ROS production. The ROS produced includes hydrogen peroxide (H$_2$O$_2$) and the superoxide radical (O$_2$•−), resulting from reduction of oxygen during the oxidative phosphorylation process in the mitochondrial respiratory chain. However, H$_2$O$_2$ and O$_2$•− are not highly reactive. H$_2$O$_2$ further decomposes to form the highly reactive species, hydroxyl radicals (·OH) (Cookson and Shaw, 1999). Similarly, O$_2$•− will react with NO• to form the highly reactive peroxynitrite radicals (ONOO•) (Beckman et al., 1994). ·OH and ONOO• are able to react with proteins, lipids and nucleic acids to cause the oxidative damage.

In living cells, there are antioxidant defense systems to protect the cells from oxidative damage. Some enzymes can remove the harmful ROS such as superoxide dismutase, catalase, glutathione, and peroxidase.

The basis of the oxidative stress is that toxicity is caused by aberrant oxidative chemistry of the active copper and zinc sites of the misfolded SOD1 enzyme. One hypothesis was that mutant SOD1 may have greater access of abnormal substrates, e.g. react with its normal reaction product, hydrogen peroxide to yield the highly
reactive hydroxyl free radical (OH\(^-\)). Another hypothesis was that reduction of bound zinc may allow Cu\(^{1+}\) SOD1 to convert O\(_2\) to superoxide, which then may react with nitric oxide (NO) within the active site to form highly reactive peroxynitrite (\(^{\cdot}\)ONOO\(^-\)) leading to nitration of tyrosine residues (J. S. Beckman et al., 1994; A. G. Estevez et al., 1999). Conflicts are found in animal studies of both peroxidase activity and peroxynitrite formation hypothesis. The original hypothesis of oxidative stress, that of aberrant copper chemistry mediated by mutant SOD1 causing ALS was called into question by the following two studies. First, deletion of a specific copper chaperone for SOD1 (CCS) in transgenic mice expressing three different mutations (SOD1G37R, SOD1G85R, and SOD1 G93A), which significantly reducing the copper loading onto SOD1 and resulting in marked activity loss of SOD1, does not affect the onset or progression of disease (Subramaniam et al., 2002). In addition, transgenic mice expressing a SOD1 variant that mutates the four histidine residues, which are responsible for holding the copper ion in the active site, produced a fully inactive protein, but still caused a motor neuron disease (Wang et al., 2003).

However, an increasing body of evidence implicates the involvement of oxidative stress in ALS. Markers of oxidative stress are significantly elevated in postmortem ALS spinal cord and motor cortex, and as well as in the cerebrospinal fluid (CSF) of ALS patients (R. J. Ferrante et al., 1997). In sporadic ALS patients, protein carbonyl levels were found to be increased in both the lumbar spinal cord and motor cortex. Oxidative damage markers for protein or lipid showed elevated immunoreactivity in motor neurons, microglia and astrocytes as well (P. J. Shaw et al., 1995; R. G. Smith et al., 1998; E. P. Simpson et al., 2004). 8-hydroxy-2’-deoxyguanosine (8-OHdG), a biomarker
of oxidatively damaged DNA, was found to be significantly increased in both the ventral horns of the cervical spinal cord and in the CSF of ALS patients (Bogdanov et al., 2000). Increased immunoreactivity to 3-nitrotyrosine was observed in the ventral horn motor neurons.

Increased oxidative damage to protein, DNA, lipid was also reported in ALS mutant SOD1 transgenic mouse models. Hall et al. investigated the extent of lipid peroxidative damage in the spinal cords of the SOD1 (G93A) mice over their life span (Hall et al., 1998), and observed that compared to non transgenic littermates, the SOD1 (G93A) mice show higher levels of lipid peroxidation over the 30-120 day time span, but the greatest motor neuronal lipid peroxidative injury is associated with the active phase of disease progression (100-120 days). Andrus et al. (1998) studied protein oxidation and observed a similar phenomenon that the greatest protein oxidation occurs between 100 and 120 days of age (Andrus et al., 1998). Further, an increase of DNA oxidation also occurs during symptomatic stage (Liu et al., 1999). On the other hand, free 3-tyrosine level was found increased in SOD1 (G93A) and SOD1 (G37R) transgenic mice compared to SOD1 (WT) transgenic mice, but the protein -bound nitro tyrosine was found unchanged (L. I. Bruijn et al., 1997a; R. J. Ferrante et al., 1997; J. Wang et al., 2003).

All these evidence suggests an involvement of oxidative stress in ALS. Whether oxidative stress is the primary cause of the disease or merely a consequence of the pathogenesis is not clear. Oxidative stress might be not the primary cause of the disease but it and other mechanisms all contribute to the progression of the ALS to some extent.
1.2 RNA oxidation and Neurodegeneration

1.2.1 RNA is more susceptible to oxidative damage

Increasing evidence shows that oxidative damage is involved in the pathogenesis of neurodegenerative disease including AD, PD, and ALS, which are all related to aging. Protein oxidation, lipid peroxidation and DNA oxidation in these neurodegenerative diseases have been reported by many groups. Compared to DNA, single-stranded RNA, lacking hydrogen bond protection, and is relatively abundant and has widely distributed within cells, probably with less protein protection as well, thus is more susceptible to be oxidatively damaged. Reactive Oxygen Species can hydroxylate guanine to produce 8-oxo-7, 8-dihydroguanosine (8OHG) in RNA and 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8OHdG) in DNA (Kasai et al., 1991); these compounds can serve as sensitive biomarkers for oxidative damage (E. S. Fiala et al., 1989; W. G. Wamer et al., 1997).

Hofer T et al. (2006) using an HPLC/ECD system demonstrated that the degree of oxidation is greater in RNA oxidation than in DNA, in isolated RNA and DNA exposed to the Fenton reaction (in vitro oxidation). They also investigated whether this happened in vivo and found that only RNA and not DNA showed a significant increase in oxidation after injection of oxidant generator into the rat liver (T. Hofer et al., 2006). The fact that RNA is more susceptible to oxidative damage compared to DNA was supported by other studies on nonneuronal cell lines including skin fibroblasts (W. G. Wamer et al., 1997), human lung epithelial cells (Hofer et al., 2005), and rat liver cells (Fiala et al., 1989).
1.2.2 Sources of reactive oxygen species (ROS) responsible for RNA oxidative damage

Due to a high metabolism rate in the central nervous system, neurons within CNS are constantly exposed to free radicals created by internal cellular metabolisms. The most common cellular free radical is superoxide radical (O$_2^-$), which is produced when an oxygen molecule gains one electron from another molecule. Excessive amount of O$_2^-$ leads to tissue damage by promoting highly reactive hydroxyl radical (OH) formation from hydrogen peroxide (H$_2$O$_2$) in the presence of iron, known as the Fenton reaction. Brain and spinal cord tissues have much higher lipid content, as compared to other tissues. Polyunsaturated fatty acids are particularly vulnerable to free radical attack, as ROS (e.g. OH$^-$), can easily attack the double bonds within membrane lipids. Free radicals can also oxidize proteins and nucleic acids (e.g., DNA), leading to subsequent cell death by way of necrosis or apoptosis. For RNA oxidation, considering RNA widely is distributed in the cell, the damaged is most likely to be caused by a more permeable and highly active form of ROS, -OH$^-$.

1.2.3 RNA oxidation and neurological disease

Several studies have shown a significant increase of 8OHdG and 8OHG levels in the brains and cerebrospinal fluid of patients with AD (S. P. Gabbita et al., 1998; M. A. Lovell and W. R. Markesbery, 2001). Nunonmura et al. (1999) used an in situ approach with monoclonal antibodies 1F7 or 15A3, which recognizing oxidized nucleosides to investigate whether nuclear and mitochondrial DNA as well as RNA is damaged in AD, and found that increased RNA oxidation is restricted to vulnerable neurons in AD, and the subcellular localization of RNA oxidation, showing cytoplasmic
predominance, is consistent with their hypothesis that mitochondria may be a major source of reactive oxygen species that cause oxidative damage in AD (Nunomura et al., 2006). The same group investigated the relationship between neuronal 8OHG, nitrotyrosine and histological alterations, i.e. amyloid-beta (A beta) plaques, neurofibrillary tangles (NFT), and extent of dementia. Their results indicated that oxidative damage is quantitatively significant early in the disease and reduces with disease progression.

These findings raise several interesting questions. Whether mRNA in the AD brain is oxidized? If mRNA in AD brain is oxidized, then is the damage random or is selective for some specific mRNA species? What would be the biological consequences of those oxidized mRNA? Will they be normally translated? To answer these questions, our lab developed a novel immunoprecipitation procedure to isolate and identify oxidized mRNA species in AD. We found that significant amounts of poly(A)+ mRNAs are oxidized in AD brains. Furthermore, we show that RNA oxidation is not random but highly selective, while no common tertiary structure or motif was identified. Most importantly, many identified oxidized mRNA species are related to AD including p21 ras, MAPK kinase 1, carbonyl reductase, SOD1, apolipoprotein D. Quantitative analysis revealed that some mRNA species are more susceptible to oxidative damage. We also investigated the biological consequence of oxidatively damaged mRNAs by expressing them in cell lines. Our data indicate that abnormal translation of corresponding proteins is observed for the oxidized mRNAs. This may implicate the potential contribution of RNA oxidation in the pathogenesis of AD (Shan et al., 2003).

Two independent groups studied whether rRNA has also been oxidized, and
found that in AD brain, rRNA, the most abundant form of RNA, also contains 8(OH) G.

RNA oxidation also has been found linked to other forms of neurological diseases, including Parkinson’s disease, Down’s syndrome, Creutzfeldt-Jakob disease, Dementia with Lewy bodies, Xeroderma pigmentosum (group A), Gerstmann-Straussler-Scheinker disease (A. Nunomura et al., 1999a; M. Guentchev et al., 2002; T. Abe et al., 2003). Notably, for both AD and Creutzfeldt-Jakob disease increased RNA oxidation was observed in both sporadic and familial cases (M. Guentchev et al., 2002; A. Nunomura et al., 2004; R. B. Petersen et al., 2005). In some animal models or experimental conditions, e.g. aging rat brain, increased RNA oxidation has also been identified. The summary of these findings was listed in Table 1.3.

There is increasing evidence suggest that RNA oxidation is a relative early pathological change during the disease progression. Greater RNA oxidation has been found parallel to the disease stages showing moderate clinical symptoms or other early pathological changes. In the severe cases of disease, RNA oxidation increased is not significant. In AD postmortem brains and subacute sclerosing panencephalitis postmortem brains, RNA oxidation is found more prominent in cases with shorter disease duration (A. Nunomura et al., 2001; M. Hayashi et al., 2002). In contrast, lipid peroxidation is observed in cases with longer disease duration. In Alzheimer’s brain, RNA oxidation is markedly increased in the hippocampal neurons, which are not afflicted by neurofibrillary tangles, or also in the cases with lower amounts of Aβ plaque deposition. Additionally, it has been reported that RNA precedes Aβ plaque deposition in a series of Down syndrome brain with early stage AD neuropathology (Nunomura et al., 1999). Increased RNA oxidation is found in the brains of patients with mild
cognitive impairment (MCI) but not in severe cases (Ding et al., 2005). All these evidence support that RNA oxidation is an early event in the process of neurodegeneration.
<table>
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<tr>
<td>Alzheimer’s disease</td>
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<td>hippocampus, cerebral cortex/ IEM</td>
<td>2001, Nunomura et al.</td>
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<td></td>
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<td>2002, Abe et al.</td>
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<td></td>
<td>hippocampus, cerebral cortex/ IP, IB, RT-PCR (mRNA)</td>
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<td>hippocampus/ IB, RT-PCR (rRNA)</td>
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<td></td>
<td>cerebral cortex/ IB (rRNA)</td>
<td>2005, Ding et al.</td>
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<td>Parkinson’s disease</td>
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<td></td>
<td>cerebrospinal fluid/HPLC</td>
<td>2003, Abe et al.</td>
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<tr>
<td>Down syndrome</td>
<td>cerebral cortex/ ICC</td>
<td>2000, Nunomura et al.</td>
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<tr>
<td>Dementia with Lewy bodies</td>
<td>cerebral cortex/ICC</td>
<td>2001, Guentchev et al.</td>
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<td>cerebral cortex/ICC</td>
<td>2002, Hayashi et al.</td>
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<tr>
<td>Xeroderma pigmentosum (group A)</td>
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<td>2002, Hayashi et al.</td>
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<tr>
<td>Gerstmann-Straussler-Scheinker disease</td>
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<td>Mouse model</td>
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<td>Cell Culture</td>
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<td>neurons under proteosome inhibition</td>
<td>Mixed astrocytes and neuron culture/ ICC, IB</td>
<td>2004, Ding et al.</td>
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**Table 1.4** Summary of findings of RNA oxidation in central nervous system

Note: ICC: immunocytochemistry; IB: immunoblot; IEM: immunoelectronmicroscopy; HPLC: high performance liquid chromatography; ELISA: enzyme-linked immunosorbent assay; RT-PCR: reverse transcription and polymerase chain reaction).
1.2.4 The biological consequence of RNA oxidation

8-hydroxylguanine is the most abundant form among more than 20 different types of oxidatively modified purine and pyrimidine bases. 8-hydroxylguanine within a RNA strand can be formed by oxidation of guanine base on the RNA or by the incorporation of oxidized free GTP from the cytosol. 8-hydroxyguanine can pair with both cytosine and adenine, and thus the oxidized RNA may affect the accuracy of the pairing capacity and leading to abnormal protein translation.

Our lab had investigated the biological consequence of RNA oxidation in vitro, by expressing oxidized mRNA in cell lines, and we found that oxidized mRNA causes loss of normal protein function and expression level. The defective protein coded by the oxidized mRNA has the potential to form aggregates, which is a common characteristic shared by many neurodegenerative diseases, such as Alzheimer’s disease, Amyotrophic lateral sclerosis, Parkinson’s disease, etc. More recently, our lab explored the relationship between RNA oxidation and neuron degeneration induced by various insults, including hydrogen peroxide, glutamate, and amyloid beta peptide, using cortical primary dissociated cultures. We found that oxidized bases in mRNAs can cause ribosome stalling on the transcripts, leading to decrease of protein expression (X. Shan et al., 2007). Tanaka et al. (2007) in a recent study demonstrated that the translation of oxidized mRNA in cell lines cause an accumulation of truncated polypeptides, presumably as a result of premature termination of translation or enhanced targeting of defective proteins toward a proteolytic degradation pathway (Tanaka et al., 2007). In another study, Honda et al (2005) studied the consequence of ribosomal oxidation in vitro using rabbit
Great progress has been made in the field of RNA research in recent years. RNA has far surpassed its classic intermediate messenger role between genetic code DNA and protein translation. Moreover, RNA is also a key regulator of many important physiological and pathological pathways. More and more non-coding RNAs (ncRNAs) have been found to have essential functions in many pathophysiological processes, other than as simple intermediates in between DNA transcription and protein synthesis. According to their size, they can be classified into microRNA (~20 bp), small RNA (~20-300 bp), and medium or large sized RNA (over ~300bp, up to or over 10,000). Functions of medium or large sized RNA includes gene silencing, gene transcription, DNA imprinting, RNA interference, tumor suppression, and various functions related to stress or apoptosis (Costa, 2005; Mattick and Makunin, 2006; Costa, 2007). It is not hard to image that if these non-coding RNAs are oxidized, this would compromise their normal functions in an array of physiological processes. And such effect is no longer merely impairing the protein synthesis, but also dysregulation at gene transcription level. Further elucidation of the consequence of oxidized non-coding RNA might provide new understandings of RNA oxidation, and its function on pathogenesis.

1.2.5 RNA oxidation and RNA repair

RNase catalyzed RNA degradation plays an essential role in damaged RNA metabolism. However, the well-known RNases have not been found to possess the capacity to selectively degrade oxidized RNA (Deutscher, 2006).

In *Escherichia coli*, RNA alkylation damage can be repaired by the
same enzyme used for a DNA-repair, in which AlkB and hABH3 hydroxylate the methyl group within the damaged bases. Other than alkylation damage repair, DNA repair also includes base excision, nucleotide excision and mismatch repair. These mechanisms may not be applied to RNA repair because they require a complementary strand, to fill in the blanks due to excision (Krokan et al., 2004).

For those 8-hydroxylguanine within an RNA strand oxidized indirectly by incorporation of oxidized free GTP from the cytosol, the cell may be able to defend against their formation, by removal of free oxidized 8(OH) GTP from the cytosolic nucleotide pool. Several studies reported that the oxidized nucleoside triphosphate or diphosphate can be hydrolyzed by MTH1 and NUDT5 in mammalian (MutT in *Escherichia coli*). Ishibashi et al (2005) studied the relationship between oxidative stress induced production of erroneous protein and the MutT activity in *Escherichia coli*. They found that in MutT deficient cells, the amount of production of erroneous protein induced by oxidative stress is 28-fold over normal cells, and almost back to normal upon over expression of MutT or NUDT5 (Ishibashi et al., 2005). On the other hand, in MTH1 knock out mice, RNA oxidation induced by kainic acid treatment was significantly increased compared to normal mice (Kajitani et al., 2006). In addition, the MTH1 expression level was found upregulated in some vulnerable neurons of AD (Furuta et al., 2001) and PD (Shimura-Miura et al., 1999) patients postmortem tissues, suggesting a compensatory response to oxidative damage.

For those 8-hydroxylguanine within a RNA strand formed directly by oxidation of RNA guanine, researchers have tried to find proteins which have specific binding to oxidized RNA, and may play a role in the repair and/or degradation of
oxidatively damaged RNA. Three proteins have been reported, including polynucleotide phosphorylase protein (Pnp) in *Escherichia coli*, PNP and Y box-binding protein 1 in human. Hayakawa et al. (2002) demonstrate PNP has great potential to bind to 8-OHG-containing RNA, and under oxidative stress conditions, PNP protein level was rapidly decreased. YB-1, a component of P-bodies, where mRNA is degraded, translocates into the P-bodies under oxidative stress, suggesting a potential role in oxidized RNA repair (H. Hayakawa et al., 2002).
CHAPTER 2

MESSENGER RNA OXIDATION OCCURS EARLY IN DISEASE PATHOGENESIS AND PROMOTES MOTOR NEURON DEATH IN THE MUTANT SOD1 MOUSE MODEL OF ALS

2.1 Abstract

Accumulating evidence indicates that messenger RNA (mRNA) oxidation may be associated with neuronal deterioration during the process of neurodegeneration. In the present study, we found that mRNAs are oxidatively damaged in amyotrophic lateral sclerosis (ALS) postmortem tissues, specifically in the affected areas. To understand the role of mRNA oxidation in the pathogenesis of motor neuron degeneration, we investigated RNA oxidation in a transgenic mouse expressing ALS-linked mutant Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1 (G93A)). We observed a significant increase in mRNA oxidation in the motor neurons and oligodendrocytes of the SOD1 (G93A) spinal cord at an early, pre-symptomatic stage. During the symptomatic stage, the dying motor neurons showed little mRNA oxidation, indicating that RNA oxidation is an early event, preceding motor neuron death. Identification of oxidized mRNA species revealed that some mRNA species were more vulnerable to oxidative damage, and importantly, many oxidized mRNA species have been implicated in the pathogenesis of ALS. The expression level of protein corresponding to the oxidized mRNA
species was significantly decreased, indicating that oxidative modification of mRNA causes reduced protein expression. Vitamin E could reduce mRNA oxidation, restore protein expression level and partially protect motor neurons. Increased mRNA oxidation also occurred in the pre-symptomatic stage of mice expressing other mutant SOD1, including SOD1(G37R), SOD1 (G85R), SOD1 (G127X) and SOD1 (H46R/H48Q). These studies suggest that mRNA oxidation is a common early event preceding motor neuron degeneration and is associated with motor neuron deterioration in ALS.

2.2 Introduction

The molecular mechanisms underlying mutant SOD1-linked FALS remain unclear. Many studies have shown that the mutant SOD1 toxicity to motor neurons is non-cell autonomous, i.e. mutant damage is required within both motor neurons and non-neuronal cells (Y. H. Gong et al., 2000; A. Pramatarova et al., 2001; M. M. Lino et al., 2002; A. M. Clement et al., 2003). Oxidative damage is believed to be one of the contributor to the pathogenesis of amyotrophic lateral sclerosis (ALS) (Reynolds et al., 2007). Oxidation of protein, DNA and membrane phospholipids has been demonstrated in mutant SOD1 transgenic mice (P. K. Andrus et al., 1998; E. D. Hall et al., 1998; D. Liu et al., 1999; Y. J. Patel et al., 2005). These oxidative damages significantly increase at active phase of disease progression. In the present study, we demonstrate that mRNA oxidation occurs early in disease pathogenesis in the transgenic mice expressing mutant SOD1 (G93A), and it promotes motor neuron death. The results of this study suggest that mRNA oxidation may be an important factor initiating the cascade of motor neuron neurodegeneration.
2.3 Materials and methods

Animal model

Human SOD1 (G93A) transgenic mice (B6SJL-Tg [SOD1-G93A] 1Gur with high copy number of the mutant human SOD1 gene) and human wild-type SOD1 transgenic mice (B6SJL-Tg [SOD1-G93A] 2Gur) (Gurney, 1994) were purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A). Adult same gender mice were housed 5 per cage, under 12 h dark/light cycles. Transgene was determined by PCR using genomic DNA extracted from tail biopsies. From the time when transgenic mice showed motor deficits, food and water were placed on the cage floor. Because of the ethical considerations, transgenic animals were killed when they can not right themselves within 30 sec.

Tissue collection and RNA isolation

After decapitation, spinal cords were removed and dissected rapidly. The samples were placed in individual tubes and homogenized in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA isolation was performed according to the manufacturer's protocol. After DNase I (Invitrogen, Carlsbad, CA) treatment, poly (A) \(^+\) RNAs were selected using the Oligo-tex mRNA Purification Kit (Qiagen). RNA concentration was measured by spectrophotometric ratio A260/A280. Poly (A) \(^+\) RNAs from 2 individual mice (100ug from each) were combined as an mRNA pool for Immunoprecipitation.

Tissue preparation and Electron microscopy

Mouse spinal cords were initially fixed by intracardiac perfusion with a solution of 0.1% sodium phosphate (pH7.6), 4% paraformaldehyde, and 2.5% glutaraldehyde, and postfixed in 1% osmium tetroxide, and embedded in Eponate 12 (Ted Pella, Redding, CA). One-micrometer sections were stained with toluidine blue. Motor neurons
were identified by their large size (>20 µm) and distinct nuclear morphologies in the ventral horn of the lumbar cord.

**Immunohistochemistry**

Mice were perfused transcardially with 4% paraformaldehyde after being deeply anesthetized with tribromoethanol (Avertin; 200 µl/10 g intraperitoneally). The brains and spinal cords were rapidly removed and cryoprotected with 20% sucrose for 24h. The sections were incubated in blocking solution (8% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) and 0.1% Triton X-100 in Tris-buffered saline (TBS)) for 60 min at 4°C and then in primary antibody solution overnight at 4°C. After thorough washing with TBS, the sections were then incubated with secondary antibody in TBS containing 2% NGS for 60 min at 4°C followed by thorough washing with TBS. The following primary antibodies were used in this study: rabbit anti-ubiquitin pAb (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-8OHG mAb (1:250, QED Bioscience, San Diego, CA), rabbit anti-EAAT3 pAb (1:1000), rabbit anti-S6 ribosomal protein (1:1500; Cell Signaling Technology, Danvers, MA), mouse anti-NADH-ubiquinol oxidoreductase 39kDa Subunit, (1:2000), mouse anti succinate-ubiquinol oxireductase 70kDa Subunit, (1:10,000), mouse anti-ubiquinol-cytochrome c oxidoreductase FeS Subunit, (1:2000), mouse anti cytochrome c oxidase VIb Subunit (1:750). Images were obtained using a Zeiss Axioskop 2 upright microscope and AxioVision software.

**Immunoprecipitation**

Poly (A)⁺ RNAs (1.5 µg) were incubated with 1.5 µg of anti-8OHG antibody (15A3) at room temperature for 16 hr. For negative control trials, the primary antibody was
omitted or pre-incubated with 24 ng/μl of 8OHG (Cayman Chemical, Ann Arbor, MI). Immobilized Protein L gel beads (20 μl) (Pierce, Rockford, IL) were added and incubated at 4°C for an additional 15 hr. The beads were washed three times with PBS and 0.04% (v/v) NP-40 (Roche Products). Afterward, the following items were added in the following order: 300 μl of PBS with 0.04% NP-40, 30 μl of 10% (w/v) SDS, and 300 μl of PCI (phenol: chloroform: isoamyl alcohol, 25:24:1). The mixture was incubated at 37°C for 15 min (vortexing every 5 min) and separated to aqueous phase and organic phase by spinning at 14,000 rpm for 5 min. The aqueous layer was collected and mixed with 40 μl of 3 M sodium acetate, pH 5.2, 2 μl of 5 mg/ml glycogen, plus 1 ml of 95% (v/v) ethanol. The sample was frozen at -80°C for 1 hr and centrifuged for 20 min. The pellet was washed with 75% ethanol and air-dried. It was resuspended in 14 μl of DEPC-treated H₂O₂.

cDNA synthesis and Southern blotting

Immunoprecipitated mRNAs were reversely transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Roche Products). For 15 μl of reaction mixture, 7 μl of immunoprecipitate mRNAs and 1 μl (0.75 μg) of oligo-(dT)₂₄-T₇ primer were mixed and incubated at 70°C for 10 min. After 2 min on ice, the master mix contained 7 μl of 5x first-strand buffer (Roche Products), 0.5 mM 2'-deoxynucleoside 5'-triphosphates [deoxy (d)-ATP, dCTP and dGTP], 0.13 mM 2'-deoxythymidine-5'-triphosphate, 0.03 mM digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (Roche Products), 2.5 U of RNase Inhibitor (Invitrogen), and 10 U of AMV reverse transcriptase (Roche Products). The mixture was incubated at 42°C for 90 min. Two microliters from 15 μl of digoxigenin-labeled cDNAs were used for additional detection by the Southern blotting method.
to compare the difference of cDNA quantities between SOD1 (G93A) transgenic and non-transgenic cases. cDNAs were resolved in 1% agarose gel and then transferred electrophoretically to a positively charged nylon membrane (Roche Products) using the Trans-Blot SD semidry transfer system (Bio-Rad, Hercules, CA) according to the directions of the manufacturer. Digoxigenin labeled on cDNAs was detected with a Digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Roche Products).

**Microarray hybridization**

Preparation of cRNA was performed according to the Two-cycle cRNA amplification protocol provided by Affymetrix. The immunoprecipitated oxidized mRNA was converted to cDNA using AMV reverse transcriptase and an GeneChip T7-oligo(dT) primer 5’-GGCCAGTGAATTGTATMCCTACTATAGGGAGGCGG-(dT) 24-3’ (Affymetrix, Santa Clara, CA). For the first-cycle IVT amplification of cRNA, MEGAscript T7 kit (Ambion, Inc.) was used. The cRNA is purified and cleanup by using RNeasy mini kit (Qiagen Inc., Valencia, CA, USA). Each cRNA sample was synthesized from 2 independent biological samples. Biotin-labeled cRNA was synthesized from second-cycle cDNA using an IVT Labeling Kit (Affymetrix, Santa Clara, CA). The yield of the in vitro transcription reaction was determined by product absorbance at 260 nm measured by NanoDrop ND-1000 (NanoDrop Technologies, Inc., Montchanin, DE), size of cRNA probes was evaluated by using RNA 6000 Nano LabChip Kit (Agilent, Palo Alto, CA, USA). Following labeling, samples were run experion to examine the quality (Affymetrix, Santa Clara, CA). Fragmented cRNA (15 µg) was used for hybridization to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA).
RT-PCR

Immunoprecipitated mRNAs were reversely transcribed with AMV reverse transcriptase (Roche) and Oligo-(dT)$_{24}$ primer. PCRs were performed in the presence of 3mM MgCl$_2$, 0.2mM dNTP, 0.25µM Primers and 2 U Taq DNA polymerase (Invitrogen) in 1x PCR buffer, with gene–specific primers, including Cu/Zn SOD1 F (5’-atggcgacgaagccgtgtgct) and R (5’-ctggcaaaatacagttcagagccaagaca), MAP2 F (5’-ttggctcactgacaatgctcacc) and R (5’-aatagcacctgtcagagccca-3’), MBP F (5’-agcatgcctctagtagccca) and R (5’-agtaggtgttctgtagagcagcata), Ribosomal protein S6 F (5’-tgatgctccagcaatgctcagta) and R (5’-tggctcctccattcagcaga), Cox Va F (5’-gacattgactgctgcaagggct) and R (5’-ttccacttctcaggtgct), Cyc C F (5’-tccagttatgtcattgaggtgg), PCM F (5’-aaagaacctgaaacagtgggagcc) and R (5’-ccaaatgtcacaatgagggg). A series of cycles (25, 30, 35 cycles) was performed (95°C for 30 s, specific annealing temperature to each set of primers for 45 s, and 72°C for 1 min). PCR products were visualized as single bands on 1% agarose gels stained with ethidium bromide.

Immunoblotting

Immunoblotting was performed as described previously (Guo et al., 2003). Briefly, Protein extracts were generated from spinal cord, resolved by SDS-PAGE and transferred onto PVDF membranes. The following primary antibodies were used: rabbit anti-MBP pAb (1:3000; Chemicon International, Inc, Temecula, CA), goat anti-β-Actin (1:3000; Santa Cruz, CA). The immunoreactive bands were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) according to manufacturer’s directions.
**Vitamin E treatment**

SOD1 (G93A) transgenic (n=32) mice were treated with vitamin E, and their littermates SOD1 (G93A) (n=32) and wild type mice (n=32) were used as controls. Mice were orally fed with vitamin E (200IU/each for 5 day/week), and started at age of 30-day. We did not see any significant variation of onset and survival between different genders. However, the same gender littermates of wild type, G93A, and G93A treated with vitamin E transgenic mice were used to compare their onset, survival, and pathological changes.

**Grip Strength**

An objective assessment of neuromuscular performance was obtained by measuring peak force generated using a Digital Grip Strength Meter outfitted with a Hind Limb Pull Bar Assembly (Columbus Instruments). Five repetitions were taken and the average determined for each mouse. The examiner was unaware of the genotypes of the mice during measurement. Measurements were taken twice a week once the animals reached an age of 70 days. Measurements were taken on the same day of each week and at the same time of day; furthermore, all mice were measured within 2 h of each other.

**Statistical analysis**

The quantitative data in this study were expressed as the mean ± SEM. Statistical analysis was performed using the unpaired Student's t-test and the ANOVA.
2.4 Results

2.4.1 A significant increase in RNA oxidation in the motor neurons and oligodendrocytes of the SOD1 (G93A) spinal cord at pre-symptomatic stage

Immunofluorescent staining was performed using the 15A3 antibody, which recognizes both 8-OHG and 8-OHdG, markers of oxidative damage to RNA and DNA respectively. Lumbar spinal cords from different ages of SOD1 (G93A) mice and non-transgenic littermates (n=3 per group) were examined. The results showed that the intensity of 15A3 immunofluorescence was significantly increased in 45 day-old SOD1 (G93A) mice (Fig 2.1D) and further enhanced in 60 day-old SOD1 (G93A) mice (Fig 2.1B&E), while only a very faint signal was detected in non-transgenic littermates (Fig 1A&C). 15A3 immunoreactivity was prominent in the ventral horn motor neurons, as judged by their characteristic size and morphology. In addition, a significant increase of 15A3 immunoreactivity was also observed in the white matter oligodendrocytes (Fig 2.1K&L), as identified by CC1 immunostaining (Fig 2.1M). Importantly, immunoreactivity was prominent in the cytoplasm, and was diminished greatly by the RNase treatment (Fig 2.1J), but only slightly by DNase I treatment, which indicated that cytoplasmic RNA was the major site of nucleic acid oxidative damage. Moreover, the immunoreactivity was diminished completely when the antibody was pre-incubated with 8-OHG (Fig 2.1I), indicating that the observed immunoreactivity was specific to oxidized base 8-OHG in RNA. During the symptomatic stage, 15A3 signal intensity was decreased in 90 day-old SOD1 (G93A) mice (Fig 2.1F), and further reduced in 110 day-old SOD1 (G93A) mice (Fig 2.1G). The dying motor neurons showed less 15A3 immunoreactivity, while the glial cells began to show 15A3 immunoreactivity. At end stage, glial cells
showed significant 15A3 immunoreactivity (Fig. 2.1H). Figure 2.2P shows the statistical analysis of motor neuron 15A3 immunofluorescence intensity. We also examined brain sections from 60 day- and 90 day-old SOD1 (G93A) mice and non-transgenic littermates, and no increase of 15A3 immunofluorescence was observed (Fig 2.1N&O). These results indicated that RNA oxidation occurs as early as 45 days of age, progressively increasing with age, until it peaks at 60-70 days of age, when the motor neurons still look healthy, and then diminishes when the motor neurons began to degenerate.
Figure 2.1 Significantly increased RNA oxidation in the motor neurons and oligodendrocytes of the SOD1$^{G93A}$ spinal cord at presymptomatic stage. Lumbar spinal cord sections from different ages of SOD1$^{G93A}$ mice (G93A) and non-transgenic littermates (WT) were immunolabeled with 15A3 antibodies. The intensity of 15A3 immunofluorescence in SOD1$^{G93A}$ mice was significantly increased at 45 days of age (D), further enhanced at 60 days of age (B, E), and then diminishes during the symptomatic stage (F, G, H), while only a very faint signal was detected in non-transgenic littermates (A, C). 15A3 immunoreactivity was prominent in the ventral horn motor neurons and the white matter oligodendrocytes (K, L), as identified by CC1 immunostaining (M). No increase of 15A3 immunofluorescence was observed in the brain sections (N, O). The immunoreactivity was diminished greatly by the RNase treatment (J) and when the antibody was pre-incubated with 8-OHG (I). Scale bar, 50 µm.
Figure 2.1
2.4.2 RNA oxidation is an early event far preceding motor neuron degeneration

To further examine those motor neurons showing strong 15A3 immunoreactivity, we performed nuclear staining using chromatin-binding dye bis-benzimide (Hoechst 33342). The results showed that motor neurons with strong 15A3 immunoreactivity had normal nuclear and chromatin morphology (Fig 2.2A, G93A 60d). At symptomatic stage (90 days), the dying motor neurons showed abnormal nuclear and chromatin morphology but less 15A3 immunoreactivity (Fig 2.2A, G93A 90d). Mitochondrial vacuolization is considered to be one of the earliest molecular pathological changes in SOD1 (G93A) mice. We examined mitochondrial morphology by electron microscopy and the results showed that motor neurons with strong 15A3 immunoreactivity had only minor mitochondrial vacuolization (Fig 2.2B). The ubiquitinated protein aggregation is a hallmark of degenerating motor neurons. We did not observe ubiquitinated protein aggregation in motor neurons with strong 15A3 immunoreactivity at age of 60 days (Fig 2.2C). These results indicate that those motor neurons showing RNA oxidation still appear to be healthy, and that RNA oxidation is an early event.
Figure 2.2 RNA oxidation is an early event preceding motor neuron degeneration. Lumbar spinal cords from indicated age of SOD1\textsuperscript{G93A} mice (G93A) and non-transgenic littermates (WT) were examined. (A) Motor neurons with strong 15A3 immunoreactivity had normal nuclear and chromatin morphology by Hoechst 33342 staining; the dying motor neurons showed abnormal nuclear and chromatin morphology but less 15A3 immunoreactivity. The ventral horn area is shown. Arrows point the same neuron. Scale bar, 50 \(\mu\)m. (B) Motor neurons with strong 15A3 immunoreactivity had only minor mitochondrial vacuolization as examined by electron microscopy. Scale bar, 0.5 \(\mu\)m. (C) Motor neurons with strong 15A3 immunoreactivity did not have ubiquitinated protein aggregation.
Figure 2.2 continued

B

WT, 60 d  G93A, 60 d

C

15A3  Ubiquitin

G93A, 60 d  G93A, 140 d
2.4.3 Significant amounts of poly(A)+ mRNAs are oxidized

What RNA species were oxidatively damaged? We previously developed an immunoprecipitation (IP) procedure (Figure 2.3A) to isolate oxidized RNA (Shan et al., 2003) and found that up to 50% of poly(A)+ mRNAs are oxidized in Alzheimer’s disease brains (Shan and Lin, 2006). We used this established procedure to investigate whether mRNAs are oxidized in SOD1 (G93A) mice. Whole spinal cords from 60 day-old SOD1 (G93A) mice, non-transgenic littermates, and age-match transgenic mice overexpressing wild-type SOD1 (SOD1 (WT)) were examined (n=3 per group). Poly(A)+ mRNAs were isolated from each sample, and oxidized mRNAs were separated from non-oxidized mRNAs by immunoprecipitation with 15A3 antibodies. Both oxidized and non-oxidized mRNA pools were then reversely transcribed to cDNAs. DIG labeled-dUTPs were incorporated into cDNAs, so as to facilitate analysis by Southern blotting. As shown in Figure 2.3B, significant amounts of mRNAs (~30% of total spinal cord mRNA) were immunoprecipitated in SOD1 (G93A) samples while very little amounts of mRNAs were immunoprecipitated in non-transgenic samples. About 3-5% of total spinal cord mRNA was immunoprecipitated in SOD1 (WT) samples. A no-antibody control and an 8OHG-blocked antibody control were also carried out to confirm that the precipitated mRNAs were specific to 15A3. We also examined oxidized mRNA level at end stage, and found that it is about 10% of total spinal cord mRNA were oxidized. A similar immunoprecipitation procedure was also performed to examine whether ribosomal RNA is oxidized, and the results showed that only small amounts of ribosomal RNAs were immunoprecipitated (data not shown).

Furthermore, we examined whether mRNA oxidation occurs in other
mutant SOD1 transgenic mice at pre-symptomatic stage, including SOD1 (G37R) (2 month-old), SOD1(G85R) (4 month-old), SOD1 (G127X) (5.5 month-old) and SOD1 (His46R/His48Q) (6 month-old). Southern blot analysis of 15A3-immunoprecipitated mRNAs prepared from the spinal cords showed that SOD1 (G127X) and SOD1 (His46R/His48Q) had similar level of oxidized mRNA as SOD1(G93A), while SOD1 (G37R) and SOD1 (G85R) had less oxidized mRNAs compared to SOD1 (G93A) (Figure 2.3C). These results suggest that mRNA oxidation may be a common early event preceding motor neuron degeneration in ALS.
Figure 2.3 Significant amounts of mRNAs are oxidized in the pre-symptomatic stage of mutant SOD1 mice. (A), Immunoprecipitation procedure. (B) Southern blot analysis of oxidized (O) and non-oxidized (N) mRNA pools prepared from spinal cords of indicated mice (60 day-old, n=3). About 30% of total spinal cord mRNA was oxidized in SOD1^{G93A} mice (G93A) while very little amounts of mRNAs were oxidized in non-transgenic mice (WT). About 3-5% of total spinal cord mRNA was oxidized in SOD1^{WT} mice (WtSOD1). No mRNA was precipitated in the SOD1^{G93A} sample when 15A3 antibodies were pre-incubated with 8OHG (Ab block). Less amount of spinal cord mRNAs were oxidized (~10% or less) in the end stage of SOD1^{G93A} mice compared to the pre-symptomatic stage (2 months). (C) RNA oxidation occurred in the pre-symptomatic stage of different mutant SOD1 mice. Oxidized mRNA pools were prepared from the spinal cords of indicated mutant SOD1 mice (n=3). The ages of tested mice (Age) and the age of onset (Onset) are noted. Right panel shows densitometry analysis of Southern blot results.
Figure 2.3
Figure 2.3 continued

B

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C

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<th></th>
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Age | 2 | 2 | 4 | 2 | 5.5 | 6 |
Onset | N/A | 3 | 8 | 7 | 15.5 | 7 |
(months) (months) |

Intensity

G93A | G85R | G37R | G127X | H46R/H8Q
2.4.4 Some mRNA species are more susceptible to oxidative damage

To identify oxidized mRNA species, we performed DNA microarrays using Affymetrix GeneChip Mouse Genome 430 2.0. This array can analyze ~39,000 transcripts. Three arrays were performed, and in each array, oxidized mRNAs were prepared from two spinal cords of 60-day old SOD1\textsuperscript{G93A} mice so a total of six mice were analyzed. A total of 3,409 mRNA species were identified, and these oxidized mRNA species and their relative signal intensities on the chips were similar among the three arrays. Semi-quantitative PCR analysis was then performed to verify that the identified mRNA species were present in the oxidized mRNA pool. As shown in Figure 2.4A, ribosome protein S6, cytochrome c oxidase Va, cytochrome c and myelin basic protein (MBP) mRNAs, which had strong signal intensities on the arrays, were present in the oxidized mRNA pool. Microtubule-associated protein 2 (MAP-2) and pericentriolar material 1 (PCM-1) mRNAs, which had very weak signal intensities on the arrays, were hardly detected in the oxidized mRNA pool. The signal intensities of PCR products were quite consistent with the signal intensities of the oxidized mRNA species on the arrays.

We then used the computer program Onto-Express (http://vortex.cs.wayne.edu/projects.htm#Onto-Express) to analyze the identified oxidized mRNA species and grouped them according to cellular component or molecular function (Khatri et al., 2002). The analysis based on the cellular component showed that mRNA species encoded for proteins that localized to mitochondria, ribosome and cytosol were more susceptible to oxidation. The analysis based on the molecular function showed that mRNA species encoded for proteins that are involved in mitochondrial electron
transport, protein biosynthesis, myelination, protein folding and degradation, cytoskeleton, tricarboxylic acid cycle and glycolysis, were more susceptible to oxidation.

The signal intensities of the oxidized mRNA species on the chips ranged from ~200 to ~12,000. There were 60 mRNA species whose signal intensity was above 5,000, 298 mRNA species whose signal intensity was between 2,000 and 5,000, 648 mRNA species whose signal intensity was between 1,000 and 2,000, and 2,403 mRNA species whose signal intensity was below 1,000. We considered that those mRNA species whose signal intensity was above 2,000 were highly oxidized. Table 1 shows the list of these mRNA species grouped by their function (uncharacterized genes are not listed). Importantly, most of these oxidized mRNAs appear to be related to the pathogenesis of ALS. Several highly oxidized mRNA species correspond to genes linked to familial ALS or ALS-like human motor neuron disease, including SOD1, dynactin 1, and vesicle-associated membrane protein 1.

These array results indicated that some mRNA species were more susceptible to oxidative damage, which is consistent with our previous observations in Alzheimer’s brains (Shan et al., 2003) and cultured primary neurons under oxidative stress (Shan et al., 2007). Thus, RNA oxidation is not random, but highly selective. β-actin and MAP-2 mRNAs are highly abundant mRNA species, but only very small amounts of β-actin and MAP-2 mRNAs were oxidized, indicating selective mRNA oxidation was not due to the abundance of mRNA species. We also examined whether selective mRNA oxidation was due to the up-regulation of mRNA expression. Quantitative RT-PCR analysis revealed that those highly oxidized mRNA species, such as ribosome protein S6, cytochrome c oxidase Va, cytochrome c and myelin basic protein (MBP) mRNAs, were not
up-regulated in SOD1⁰⁹³A spinal cords compared to non-transgenic spinal cords (Fig. 2.4B). However, the up-regulation could exist within specific cell types, e.g. motor neurons.

Furthermore, the above described 15A3 immunofluorescent staining (Fig 2) indicated that at pre-symptomatic stage, increased RNA oxidation primarily occurred in motor neurons and oligodendrocytes, not in glial cells. The array results showed that glial cell specific mRNA species such as excitatory amino acid transporter 2 (EAAT2) and glial fibrillary acid protein (GFAP) mRNAs, which are very abundant in the glial cells, were not present in the oxidized mRNA pool. This indicates the specificity of the identified oxidized mRNA species.
Figure 2.4 Semi-quantitative RT-PCR analysis of oxidized mRNA. (A) Semi-quantitative RT-PCR analysis confirmed that the identified mRNA species by DNA microarray were present in the oxidized mRNA pool. Myelin basic protein (MBP), cytochrome c, cytochrome c oxidase Va and ribosome protein S6 mRNAs, which had strong signal intensities on the arrays, were present in the oxidized mRNA pool. Microtubule-associated protein 2 (MAP-2) and pericentriolar material 1 (PCM-1) mRNAs, which had very weak signal intensities on the arrays, were hardly detected in the oxidized mRNA pool. These oxidized mRNA species are significantly decreased in vitamin E treated SOD1^{G93A} mice (G93A+vitE). (B) Semi-quantitative RT-PCR analysis showed that the oxidized mRNA species are not upregulated in the whole spinal cord of SOD1^{G93A} mice compared to their non-transgenic littermates. SOD1 mRNA, which include endogenous mouse SOD1 and transgenic human SOD1, was used as a control.
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Table 2.1 highly oxidized mRNA species in the spinal cord of 60-day SOD1 (G93A) transgenic mice
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**ATP biosynthesis**

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<td>AV172216</td>
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**Sodium-potassium-exchanging ATPase activity**

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<td>AV152334</td>
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**Electron transport chain**

**Complex I**

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**Complex IV**

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**ATP Synthase (Complex V)**

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<td>NM_007512</td>
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**Adenine Nucleotide Translocator**

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**oxidoreductase activity**

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<td>BG060909</td>
<td>stearoyl-Coenzyme A desaturase 2</td>
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<td>AK009462</td>
<td>aldo-keto reductase family 1, member A4 (aldehyde reductase)</td>
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<td>AV018774</td>
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<td>NM_010239</td>
<td>ferritin heavy chain</td>
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<td>NM_008133</td>
<td>glutamate dehydrogenase</td>
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<td>AV300942</td>
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**TCA cycle**

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Table 2.1 continued

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<td>BC008184</td>
<td>aldolase 3</td>
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<td>NM_013509</td>
<td>enolase 2, gamma neuronal</td>
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**protein folding**
- NM_022310: Heat shock 70KD protein 5 (glucose-regulated protein) | 1.4 ± 1 |
- NM_008907: peptidylprolyl isomerase A | 2.9 ± 0.9 | * |
- BM941165: cytochrome c oxidase, subunit XVII assembly protein homolog (yeast) | 1.7 ± 0.3 |
- BM210281: chaperonin subunit 6a (zeta) | 1.2 ± 0.8 |
- C77287: heat shock protein 90kDa alpha (cytosolic), class A member | 4 ± 1.3 | * |
- BC006722: heat shock protein 8 | 1.8 ± 1.3 |
- AV038603: FK506 binding protein 1a | 2.2 ± 0.5 |
- NM_009836: chaperonin subunit 3 (gamma) | 2.1 ± 0.8 |
- NM_009964: crystallin, alpha B | 2.1 ± 0.6 | * |

**proteasome**
- AV263662: proteasome (prosome, macropain) 26S subunit, ATPase 2 | 3.4 ± 1.2 |
- AV212146: proteasome (prosome, macropain) subunit, beta type 4 | 2.4 ± 0.4 | * |
- NM_011184: proteasome (prosome, macropain) subunit, alphatype 3 | 2.2 ± 1.3 |

**ubiquitin cycle**
- NM_019912: ubiquitin-conjugating enzyme E2D 2 | 2.9 ± 1 |
- BB315985: F-box and WD-40 domain protein 11 | 3 ± 0.9 |
- NM_019712: ring-box 1 | 3.1 ± 1.4 |
- AU080586: microtubule-associated protein 1 light chain 3 beta | 5.8 ± 4.2 |

**lysosome**
- NM_010684: lysosomal membrane glycoprotein 1 | 2.7 ± 0.7 |
- BB560429: lysosomal-associated protein transmembrane 4B | 3.8 ± 0.8 |

**calcium ion binding**
- NM_012061: Ca²⁺-dependent activator protein for secretion | 2.1 ± 0.9 |
- BF608828: Mus musculus tumor protein, translationally-controlled 1 (Tpt1) | 4.1 ± 1.2 | * |
- NM_010097: Sparc-like1 | 2.6 ± 1.3 | * |
- NM_012038: visinin-like 1 | 3.4 ± 1.1 | * |
- NM_016760: clathrin, light polypeptide | 2.3 ± 0.3 |
- BG067649: epidermal growth factor receptor pathway substrate 15 | 2 ± 1.1 | * |
- NM_009722: ATPase, Ca++ transporting, cardiac muscle, slowtwitch 2 | 3.3 ± 1 | * |
- BC021347: calmodulin 2 (phosphorylase kinase, delta) | 6 ± 1.1 | * |

Continued
Table 2.1 continued

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Note: Intensity index equals to the average of three arrays divided by 1000. SD means standard deviations among three arrays.

* The mRNA species are also highly oxidized in human ALS array

** Many subunits of electron transport chain are also highly oxidized in SOD1 (G93A) transgenic mice, but they may have a different name.
2.4.5 Some proteins corresponding to oxidized mRNA species are decreased

Our previous studies indicate that oxidative modification of mRNA causes reduced protein expression (X. Shan et al., 2003; X. Shan et al., 2007). We examined protein expression levels for the oxidized mRNA species. Since the oxidized mRNAs primarily occur in motor neurons, which are a small portion of total spinal cord cell population, immunoblot analysis was not sensitive enough to detect the change, so immunofluorescent staining was performed. The results showed that some proteins, whose mRNAs were highly oxidized, were significantly decreased in the motor neurons of 60 day-old SOD1 (G93A) lumber spinal cords. For example, the protein levels of cytochrome c oxidase VIb (Fig. 2.5A, *Cox VIb*) and NADH-ubiquinol oxidoreductase subunit 39kDa (Fig. 2.5B, *NADH oxi*) were decreased. On the other hand, the neuronal glutamate transporter EAAT3, whose mRNA was not oxidized, was not decreased. However, not every tested proteins corresponding to oxidized mRNA species were decreased as examined by immunofluorescent staining. For example, the protein levels of ribosomal protein S6, neurofilament L, or tubulin β, whose mRNAs were oxidized, were not decreased. It is possible that immunofluorescent staining can only detect the dramatic change. Further, the white matter oligodendrocytes also showed significant mRNA oxidation in SOD1 (G93A) mice (Fig. 2.1). Myelin basic protein (Fig. 2.5C, MBP), whose mRNA was oxidized, was decreased as determined by immunoblotting. These results indicated that some proteins corresponding to oxidized mRNA species are decreased.
Figure 2.5 Proteins corresponding to oxidized mRNA species are decreased. (A & B) Immunofluorescent staining of lumbar spinal cords sections prepared from indicated mice shows that protein levels of cytochrome c oxidase VIb (Cox VIb) and NADH-ubiquinol oxidoreductase subunit 39kDa (NADH oxi), whose mRNAs were highly oxidized, were decreased in SOD1 (G93A) mice, but partially restored in the vitamin E treated mice. EAAT3 protein, whose mRNA was not oxidized, was not decreased. Statistic analysis of immunoreactivity within motor neurons (n=20) is shown. *P<0.01 (C) Immunoblot analysis shows that myelin basic protein (MBP), whose mRNA was oxidized, was decreased. Densitometry analysis of immunoblot is shown (n=3). *P<0.01, **P<0.001
2.4.6 Vitamin E reduces mRNA oxidation in SOD1 (G93A) mice

We investigated whether mRNA oxidation contributes to the pathogenesis of the disease. We first tested if vitamin E would reduce mRNA oxidation. SOD1 (G93A) mice were orally fed with vitamin E (200 IU/day per mouse, 5 days/week) at age of 30-day (n=3 per group). The dosage of vitamin E was chosen based on high dose vitamin E therapy in ALS patients (M. Graf et al., 2005). After 30 days of treatment (at age of 60-day), lumbar spinal cords were harvested for analysis. Immunofluorescent staining (Fig. 2.6A) and immunoprecipitation analysis (Fig 2.4A, compared G93A with G93A+vitE in oxidized mRNA pool) revealed that mRNA oxidation level was significantly decreased in the treated mice. Importantly, the protein expression levels for cytochrome c oxidase VIb (Fig. 2.5A, Cox VIb), NADH-ubiquinol oxidoreductase subunit 39kDa (Fig. 2.5B, NADH oxi), and myelin basic protein (Fig.2.5C, MBP) were elevated in the treated mice (G93A + vit E) when compared to the non-treated mice (G93A). These results indicated that vitamin E treatment can reduce mRNA oxidation in SOD1 (G93A) mice.

2.4.7 Reduced mRNA oxidation by vitamin E delays disease onset, and improves motor performance, but does not change survival

We next investigated if the reduction of mRNA oxidation by vitamin E would prevent or delay the progression of ALS. The SOD1 (G93A) mice were orally fed with vitamin E started at age of 30-days until they reached the end-stage of disease. Motor performance was monitored by measuring grip strength. We found there was a 23% decline in grip strength in the non-treated mice (n=17) but only an 8% decline in the treated mice at 90 days (n=17); there was a 60% decline in the non-treated mice and a 40% decline in the treated mice at 110 days (Fig. 2.6B). The cumulative
probability of onset, as defined by the grip strength deficit, was significantly delayed (by ~15 days, \( p<0.01 \)) in the treated mice (106±6.1 days, \( n=17 \)), compared to the non-treated mice (91±4.3 days, \( n=17 \)) (Fig. 2.6C). However, the mean life span was not significantly different between the treated mice (123±7.6 days, \( n=12 \)) and the non-treated mice (122±7.3 days, \( n=13 \)) (Fig. 2.6D). The disease duration, as defined the period from the time of 50% decline in grip strength to the time of death, was significantly shortened in the treated mice (10±4.8 days), compared to the non-treated mice (17±6.6 days, \( p<0.01 \)) (Fig. 2.6E). These results indicated that decreased mRNA oxidation by vitamin E can delay disease onset, which is consistent with the previous study by Gurney et al. (Gurney et al., 1996).
Figure 2.6 Vitamin E reduces RNA oxidation and delays the disease onset, but not the survival. (A) Immunofluorescent staining of lumbard spinal cords sections prepared from indicated mice shows that RNA oxidation is significantly decreased in vitamin E treated mice (n=3 per group). 15A3 antibodies were used to detect oxidized RNA. (B & C) The decline in motor performance by measuring grip strength was significantly delayed (~14 days, *P<0.005) in the vitamin E treated mice. Statistic analysis of immunoreactivity within motor neurons (n= 20) is shown. *P<0.01 There was no significant difference in the onset of paralysis or life span between the vitamin E treated and non-treated mice (D). The disease duration is significantly shortened (*P<0.01) (E).
Figure 2.6 continued
Figure 2.6 continued

D

Age (days)

Probability of survival

80 90 100 110 120 130 140 150

E

Disease duration (days)

G93A

G93A + vit E

- 68 -
One striking observation in the above onset study (Fig. 2.6) was that at age of 100-days, the non-treated mice exhibited grip strength deficit and were sick while the treated mice still had normal motor performance and were still very active. However, between 110 and 120 days of age, the treated mice developed onset rapidly and died within a similar age range as the non-treated mice. We therefore decided to examine the pathological changes at 100 days and 120 days of age. To examine the numbers and morphology of the motor neurons, we performed cresol violet staining on the lumbar spinal cord sections from non-treated and treated mice (n=3 per group). The results showed that at 100 days of age, lots of motor neurons were lost in the non-treated mice, and the remaining motor neurons were atrophic; in contrast, there was less motor neuron loss in sections from the treated mice, and the motor neurons were healthier in appearance, compared to the non-treated mice (Fig. 2.7A). However, at 120 days of age, there was no obvious difference between treated and non-treated mice samples, in that the majority of motor neurons had degenerated (not shown). We also performed toluidine blue staining (Fig. 2.7A), and the results were consistent with the cresol violet staining results. Importantly, there was significant vacuolization in the non-treated mice and only a slight vacuolization in the treated mice at 100 days of age. These results clearly indicated that vitamin E can protect motor neurons from SOD1 (G93A)-mediated degeneration up to 100 days of age. Notably, significant protein oxidation (Andrus et al., 1998) and lipid peroxidation (Hall et al., 1998) occurs between 100 and 120 days of age; thus, the observed protective effects by vitamin E is primary due to reduction of RNA oxidation.
We examined the effects of vitamin E on the occurrence of the following events associated with SOD1 (G93A) mice: gliosis, ubiquitin aggregation, and mitochondria vacuolization. At 100 days of age, both GFAP immunostaining, a marker for reactive gliosis, and ubiquitin immunostaining, a marker for protein aggregation, were significantly reduced in the treated mice, compared to the non-treated mice (Fig. 2.7B). However, at 120 days of age, no obvious difference was observed (not shown), suggesting that these are probably secondary events to motor neuron degeneration. Further, we performed electron microscopy to examine mitochondrial morphology of motor neurons. As shown in Figure 2.7C, severely swollen mitochondria with disorganized and dilated cristae were observed in the motor neurons of 75 day-old SOD1 (G93A) mice. Significantly, the treated mice exhibited almost normal mitochondrial morphology at 75 days, and even at 102 days, the mitochondria only slightly vacuolated, and the cristae were clear, not severely damaged. These results suggest that mitochondria vacuolization may be highly associated with RNA oxidation. One important data supporting this possibility is that as described above, many mRNAs encoded proteins involved in mitochondrial functions were highly oxidized (Table 2.1).
Continued

**Figure 2.7** Effect of vitamin E on the course of disease in SOD1 (G93A) mice. (A) Cresyl violet-stained and toluidine blue-stained sections through the ventral horn of lumbar spinal cord show partial preservation of neurons in vitamin E treated mice compared with non-treated mice at age of 100 days. The number of motor neurons in the lumbar spinal cord was counted \(n=3\) per group, \(*P<0.01\). Vitamin E delays the progressive loss of motor neurons. (B) Immunofluorescent staining of lumbar spinal cord sections shows that gliosis (GFAP staining) and ubiquitin aggregation (ubiquitin staining) were significantly reduced in vitamin E treated mice (100 day-old). The ventral horn area is shown. Scale bar, 10 µm. (C) Electron microscopy shows that vitamin E significantly reduces mitochondria vacuolization in motor neurons. Scale bar, 0.5 µm
Figure 2.7 continued

B

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2.5 Discussion

In the present study, we investigated whether RNA oxidation plays a role in the disease progression of SOD1 (G93A) mice. The results demonstrate that there is a significant increase in RNA oxidation in motor neurons and oligodendrocytes of SOD1 (G93A) spinal cord at early pre-symptomatic stage (45-60 days). At symptomatic stage (90-110 days), the dying motor neurons show little RNA oxidation. Identification of oxidized RNA species reveals that significant amounts of poly(A)$^+$ mRNAs are oxidized and some mRNA species are more susceptible to oxidation damage. Investigation of proteins corresponding to oxidized mRNA species reveals that their expression levels are decreased. Furthermore, reduced mRNA oxidation by vitamin E delays disease onset, ubiquitin aggregation, gliosis, and significantly reduces mitochondria vacuolization. This study indicates that mRNA oxidation is an early event far preceding motor neuron degeneration, and it may contribute to the pathogenesis of the disease.

Oxidative damages have been previously studied in SOD1 (G93A) mice. Hall et al. investigated the extent of lipid peroxidative damage in the spinal cords of the SOD1 (G93A) mice over their life span (Hall et al., 1998), and observed that compared to nontransgenic littermates, the SOD1 (G93A) mice show higher levels of lipid peroxidation over the 30-120 day time span, but the greatest motor neuronal lipid peroxidative injury is associated with the active phase of disease progression (100-120 days). Andrus et al. investigated protein oxidation and observed a similar phenomenon that the greatest protein oxidation occurs between 100 and 120 days of age (Andrus et al., 1998). Further, an increase of DNA oxidation also occurs during symptomatic stage ((Liu et al., 1999). In the present study, we found that RNA oxidation occurs as early
as 45 days progressively increases with age, until it peaks at 60-70 days of age, and then diminishes when the motor neurons begin to degenerate (Fig. 2.1). Those motor neurons showing RNA oxidation appear to be still healthy as judged by the nuclear and chromatin morphology and mitochondrial morphology (Fig. 2.2). These results indicate that RNA oxidation is an early event and suggest that RNA is more susceptible to oxidative damage than lipid, protein, and DNA. It is probably due to that RNA has less protection and repair systems. Why does RNA oxidation decrease during the disease stage (90-120 days) while the free radicals significantly increase? Kirby et al. reported a transcriptional repression in a motor neuron cell line (NSC-34 cells) stably expressing SOD1 (G93A) (Kirby et al., 2005). It is possible that reduced RNA oxidation is due to a decrease of transcription capacity in the dying motor neurons. It is also possible due to the fact that the apoptotic pathway is initiated at this time and mRNA start to degrade. Degradation of cellular mRNA is a general early apoptosis-induced event (Del Prete et al., 2002).

RNA oxidation primarily occurs in motor neurons and oligodendrocytes at pre-symptomatic stage (Fig. 2.1). This indicates that both cell types are more vulnerable to RNA oxidation. Oligodendrocytes are responsible for myelinating axons in CNS. Recent data indicate that oligodendrocytes are as vulnerable as neurons to brain ischemia and spinal cord injury. Like neurons, oligodendrocytes are highly vulnerable to injury by oxidative stress. Oligodendrocytes, compared to other cells, have a high lipid content, high iron content, low supplies of the cellular antioxidant (S. K. Thorburne and B. H. Juurlink, 1996; B. H. Juurlink et al., 1998). It has been shown that astrocytes are the primary site of the production of ROS that causes delayed neuronal death but has no effect on astrocytes themselves (Abramov et al., 2004). The resistance of
astrocytes to ROS has been ascribed to their greater antioxidant capacity compared to neurons (Oka et al., 1993). The concentration of reduced glutathione, the major intracellular antioxidant, has been found to be markly higher in astrocytes as in neurons (Huang and Philbert, 1995). However, during the disease stage (90-120 days), free radicals are significantly increased so increased RNA oxidation occurs in the glial cells (Fig.2.1 G&H).

We found that about 30% of total spinal cord mRNA is oxidatively damaged in SOD1 (G93A) mice at pre-symptomatic stage (Fig 2.3B). Considering that RNA oxidation primarily occurs in motor neurons and oligodendrocytes, which are small portion of total spinal cord cell population, it is conceivable that significant amounts of mRNAs are oxidized in these cells. Further, about 3-5% of total spinal cord mRNA is oxidized in transgenic mice overexpressing wild-type SOD1 (Fig 2.3B). This result is expected because overexpression of wild-type SOD1 leads to an increase in the levels of oxidative damage to DNA, proteins and lipids in NT-2 cells (M. Lee et al., 2001).

Increased mRNA oxidation also occurs in the pre-symptomatic stage of mice expressing other mutant SOD1 (Fig. 4B). Mutant SOD1 (G37R), like SOD1 (G93A), retain full dismutase activity (Wong et al., 1995). Both SOD1 (G85R) and SOD1 (G127X) lack dismutase activity and are unstable (Williamson and Cleveland, 1999; Jonsson et al., 2004). Mutant SOD1 (H46R/H48Q) possesses little or no SOD1 activity (Wang et al., 2002). The protein levels of mutant SOD1 in these mice are different: SOD1 (G93A), SOD1 (G37R), SOD1 (G85R) and SOD1 (G127X) mice express 17-, 5-, 0.9- and 0.45-fold human mutant SOD1 of mouse wild type SOD1, respectively (Wong et al., 1995; Bruijn et al., 1997; Jonsson et al., 2004; Jonsson et al., 2006; Julien and Kriz,
This suggests that increased RNA oxidation is a common feature in these ALS mouse models and has nothing to do with SOD1 activity (active or inactive form) or mutant SOD1 expression level.

We then identified oxidized mRNA species by DNA microarray and found that some mRNA species are more susceptible to oxidative damage; thus, RNA oxidation is not random but highly selective. This phenomenon was also observed in Alzheimer’s brains (Shan et al., 2003) and the cultured primary neurons under oxidative stress (X. Shan et al., 2007). A very striking finding in this study is that many identified known oxidized transcripts are related to ALS: (1) Genes linked to familial ALS or ALS-like human motor neuron disease. Mutations in the SOD1 gene are the most common form of inherited ALS, accounting for ~20% of all the familial ALS forms and corresponding to 1%-2% of all ALS cases (Rosen et al., 1993). A missense mutation in the p150 subunit of dynactin (DCTN1) has been described in a human kindred with a slowly progressive, autosomal dominant form of lower motor neuron disease, suggesting a possible risk factor for ALS (B. H. LaMonte et al., 2002; I. Puls et al., 2003). A dominant missense mutation in the VAMP-B (vesicle-associated membrane protein B) gene (ALS8) has been linked to an atypical ALS that is accompanied by an unusual tremor (A. L. Nishimura et al., 2004). VAMP-B interacts with VAMP-A involving in vesicular trafficking. (2) Neurofilaments. The genes encoding three neurofilament subunits have long been suspected as a causative for ALS because of their link with motor neuron pathology in mice and humans. Aberrant neurofilament accumulations are a pathological hallmark of both familial and sporadic ALS (Julien, 1997). Mice with increased levels of wild-type NF-H or NF-L subunits develop age-dependent motor neuron pathology (F. Cote et al., 1993; Z. Xu et
al., 1993), while expression of a point mutation in NF-L produces fatal, progressive paralysis (Lee et al 1994). (3) Protein degradation. Reduction of proteasome 26S function and protein chaperones activities have been found in lumbar spinal cord of SOD1 (G93A) transgenic mice at early pre-symptomatic stage (W. Bruening et al., 1999; E. Kabashi et al., 2004; H. Tummala et al., 2005). Impaired function of protein degradation pathway would enhance the aggregates formation. (4) Glycolysis and TCA. Depleted ATP levels and reduction of glucose use have been reported in spinal cords of SOD1 (G93A) mice at pre-symptomatic stage (S. E. Browne et al., 2006). Abnormal glycolysis and TCA process combined with mitochondrial electron transport chain dysfunction could result in ATP synthesis impairment. (5) Mitochondrial electron transport chain (ETC). Dysfunction of mitochondrial ETC has been previously evidenced in SOD1 (G93A) mice and ALS patients. Jung et al.(2002) examined the function of mitochondrial ETC in the spinal cords of the SOD1 (G93A) mice at different ages, and found that mitochondrial ETC activities, especially the Complex I (NADH dehydrogenase), at ventral horn area are significantly decreased, which begins prior to disease onset (Jung et al., 2002). Further, a decrease in Complex IV (COX) activity was detected in individual motor neurons in sporadic ALS patients (K. Fujita et al., 1996; G. M. Borthwick et al., 1999). Majority of mRNAs corresponding to Complex I and IV subunits are highly oxidized. It is possible that mRNA oxidation is responsible for the observed dysfunction. Furthermore, mRNA oxidation may be also responsible for mitochondria vacuolization; reduced mRNA oxidation by vitamin E significantly restores mitochondrial morphology (Fig. 2.7C). (6) Ca+ signaling pathway. Abnormal calmodulin may interfere in the normal calmodulin-dependent signaling pathway, resulting in increase of motor neuron vulnerability.
upon excitotoxic insults. (7) Metallothioneins (MTs). MTs, known to bind copper ions and decrease oxidative toxicity, have been suggested to have important roles in the pathophysiology of ALS (Smith and Lee, 2007). It has been reported that MT expression is increased in SOD1 (G93A) transgenic mice (Gong and Elliott, 2000), and when cross the SOD1 (G93A) mice with MT-I- and MT-II-deficient mice, significant earlier onset of clinical signs and death had been observed (Nagano et al., 2001). (8) Protein transport. Defective axonal transport has been found in ALS mouse model (Collard et al., 1995). Disruption of dynein/dynactin inhibits retrograde axonal transport in postnatal motor neurons cause a late-onset progressive degeneration in transgenic mice (B. H. LaMonte et al., 2002). Dominant point mutations in dynein cause motor neuron disorders have been found in both ALS patients and mouse model (M. Hafezparast et al., 2003; I. Puls et al., 2003). (9) Structural constituent of myelin sheath. Myelin basic protein (MBP) and proteolipid protein (PLP) mRNAs are highly oxidized in SOD1 (G93A) spinal cords. MBP, the second most abundant protein, after the PLP, in CNS myelin, is responsible for adhesion of the cytosolic surfaces of multilayered compact myelin (Boggs, 2006). MBP was report to be related to axon degeneration (Banik et al., 1987). Further, a proteomics study shows that MBP protein level is significantly decreased in ALS spinal cords (Ekegren et al., 2006). mRNA oxidation may be responsible for loss of MBP protein (Fig. 2.5 C). PLP is a highly abundant protein that accounts for over half the total protein in CNS myelin. It is reported that myelin PLP support the contact between oligodendrocytes and axons (D. A. Yool et al., 2001). Recent studies showed that myelin sheaths contribute to the structure and stability of the axons (L. L. Kirkpatrick and S. T. Brady, 1994; S. T. Brady et al., 1999; L. L. Kirkpatrick et al., 2001). Abnormal expressed myelin
protein may affect the axon stability and contribute to axon degeneration.

We examined protein expression levels for the oxidized mRNA species and found that some proteins corresponding to oxidized mRNA species are decreased (Fig. 2.5). Our previous studies demonstrated that oxidized bases in mRNAs can cause ribosome stalling on the transcripts, leading to decrease of protein expression (X. Shan et al., 2007). A recent proteomic analysis study (Massignan et al., 2006) shows that the expression levels of several proteins, including ATPase β chain, phosphoglycerate mutase 1, and Serpinb 1a, are significantly decreased in the spinal cords of 9-week-old SOD1 (G93A) mice, compared to their nontransgenic littermates. Interestingly, the mRNA species corresponding to these proteins are oxidized, suggesting that mRNA oxidation may account for loss of protein expression. However, not every tested protein corresponding to oxidized mRNA species was altered as examined by immunofluorescent staining. It is possible that protein level might be decreased but immunofluorescent staining was not sensitive enough to detect the change. One important data to support that oxidative modification of mRNA alters protein expression is that protein expression levels for the oxidized mRNAs are elevated in the vitamin E treated mice (Fig. 2.5). Furthermore, a recent study demonstrates that oxidized mRNA induces translation errors, producing short polypeptides because of premature termination or translation error induced degradation (Tanaka et al., 2007).

Does mRNA oxidation contribute to the pathogenesis of the disease? We approached this important question by treating SOD1 (G93A) mice with vitamin E. As described above, mRNA oxidative damage occurs at early age of mice, but significant protein, lipid and DNA oxidative damages do not occur until active disease
progression stage. Thus, the protective effects observed in the early symptomatic stage of vitamin E treated mice may be primarily due to reduced mRNA oxidation. Three observations strongly support that mRNA oxidation does contribute to the disease. First, protein expression study (Fig.2.4) showed that protein expression levels for the oxidized mRNA species are decreased, but vitamin E can reduce mRNA oxidation (Fig. 2.4A &2.6) and consequently restore normal protein expression levels (Fig.2.5). Second, onset study (Fig. 2.6) showed that the vitamin E treated mice still had normal motor performance and were still very active at age of 100-day while the non-treated mice were already sick. Although the treated mice developed onset rapidly between 110 and 120 days and died at similar time as the non-treated mice, it is probably due to that reduced mRNA oxidation by vitamin E partially diminishes toxicity in motor neuron, but toxicities from other non-neuronal cells still cause motor neuron degeneration. Third, mitochondrial morphology study (Fig. 2.7C) showed that the vitamin E treated mice exhibited almost normal mitochondrial morphology at 75 days while the non-treated mice exhibited severely swollen mitochondria with disorganized and dilated cristae. Our results indicate that mRNA oxidation is a major toxicity in motor neurons and also oligodendrocytes in SOD1 (G93A) mice. mRNA oxidation may account for many neuropathological changes reported previously, especially mitochondria dysfunction.

Dietary supplement with vitamin E is widely used in clinical as antioxidant for ALS patients. Although in previous studies vitamin E did not prolong survival of ALS patients (M. Graf et al., 2005), Ascherio et al.(2005) found that those individuals who took vitamin E supplements for 10 years had a less than half risk of death from ALS than that of vitamin E nonusers (A. Ascherio et al., 2005). In contrast, no significant
associations were found for use of vitamin C or multivitamin supplements. Our results, consistent with the Gurney et al. study, show that early administration of vitamin E delays the onset but not the survival in SOD1 (G93A) mice (Gurney 1998). In our study, similar results were obtained even we use mega-dose vitamin E. One possibility is that limited amount of vitamin E can cross the blood brain barrier by oral feeding. In addition, we did not see any side effect of mega-dose vitamin E to the mice.

In conclusion, RNA oxidation occurs early in disease pathogenesis in SOD1 (G93A) mice, and importantly, it promotes motor neuron death. From these studies, we strongly consider that oxidative stress, especially RNA damage, is a key toxicity in motor neurons as well as oligodendrocytes in the SOD1 (G93A) ALS mouse model. However, the mutant SOD1 toxicity to motor neurons is not only within motor neurons but also from non-neuronal cells.

There are still many questions need to be answered about RNA oxidation, but at this moment our data suggest that RNA oxidation itself is directly associated with neuronal deterioration instead of harmless epiphenomena during the process of neurodegeneration.
CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF OXIDIZED MESSENGER RNA SPECIES IN AMYOTROPHIC LATERAL SCLEROSIS

3.1 Abstract

Many studies have found evidence of increased oxidative stress in ALS pathogenesis. In our previous study (Chapter 2), using an ALS transgenic mouse model, overexpressing mutant Cu^{2+}/Zn^{2+} superoxide dismutase SOD1 (G93A), we have demonstrated that mRNA oxidation occurs early in disease pathogenesis in the mice, and it promotes motor neuron death. Identification of oxidized RNA species revealed that significant amounts of mRNAs were oxidized, and that some mRNA species were more vulnerable to oxidative damage, including mRNA encoding for translational machinery proteins, mitochondrial electron transport chain (ETC) components, ATP biosynthesis proteins, and protein degradation related proteins, etc. In our current study, we investigate whether RNA oxidation occurs in human ALS patients. We found that varied amounts of mRNA has been oxidized in the motor cortex and lumbar spinal cord of both sporadic and familial ALS patients, but not in normal patients or in ALS disease spared tissues. Further identification of the oxidized mRNA species, using microarray analysis, revealed that mRNA oxidation is not random but is highly selective even at the end stage of...
the disease. Compared to the pre-symptomatic stage SOD1 (G93A) transgenic mouse array, human ALS array covers the majority of oxidized mRNA species, and showed more categories of oxidized mRNAs corresponding proteins involved in apoptosis, cell cycle, immune response, transcription. Surprisingly, comparison profile of oxidized mRNA species in Alzheimer’s disease frontal cortex arrays to ALS motor cortex arrays showed that the oxidized mRNA species in these two neurodegenerative diseases at end stage are quite similar. These results indicate that mRNA oxidation is a general early pathological alteration in ALS pathogenesis or even other neurodegenerative diseases, oxidized mRNAs at early stage may participate the initiation of motor neuron degeneration, and at the end stage in which motor neurons are dying, oxidized mRNA might accelerate the neurodegeneration process.

3.2 Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset, fatal neurodegenerative disorder that is characterized by the selective loss of upper and lower motor neurons, leading to progressive weakness, muscle atrophy with eventually death within 5 years after onset. About 5% to 10% of ALS cases are familial, while the majority of cases are sporadic (Rosen et al., 1993). The etiology of ALS remains largely unknown. Numerous studies have firmly established that increases in oxidative damage occur in ALS pathogenesis. Oxidative damage is caused by imbalance between the production of potentially toxic reactive oxygen species (ROS) and a system's ability to detoxify the ROS. ROS-mediated oxidative damage in ALS, including lipid peroxidation, protein oxidation, protein nitration and DNA oxidation are elevated both in sporadic and familial ALS patients and as well as in several animal models (P. J. Shaw et al., 1995; P.
K. Andrus et al., 1998; E. D. Hall et al., 1998; D. Liu et al., 1999; E. P. Simpson et al., 2004). RNA, compared to DNA or protein, has less protection, repair system and an extensive cellular distribution, thus is much more vulnerable to oxidative damage. Increased RNA oxidation has been reported as early event in neurons in other neurodegenerative disease such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) (A. Nunomura et al., 1999b; J. Zhang et al., 1999). Recently, we investigated the relationship between mRNA oxidation and motor neuron degeneration, and observed a significant increase in RNA oxidation in the motor neurons and oligodendrocytes of the SOD1G93A spinal cord at an early, pre-symptomatic stage. Identification and characterization of oxidized RNA species revealed that some mRNA species were more vulnerable to oxidative damage. The analysis based on the molecular function showed that mRNA species encoded for proteins that are involved in hydrogen-transporting ATP synthase activity, NADH dehydrogenase activity, cytochrome c oxidase activity, structural constituent of myelin sheath, structural constituent of ribosome, L-lactate dehydrogenase activity, and antioxidant activity were more susceptible to oxidation. The analysis based on the biological process showed that mRNA species encoded for proteins that involved in S-adenosylmethionine biosynthesis, mitochondrial electron transport, tricarboxylic acid cycle, myelination, ATP synthesis coupled proton transport, chromatin modification, and response to oxidative stress, protein biosynthesis, and glycolysis were more susceptible to oxidation. The expression level of protein corresponding to the oxidized mRNA species was significantly decreased. Furthermore, vitamin E significantly reduced mRNA oxidation and partially protected motor neurons from SOD1 (G93A)–mediated neurodegeneration in mice. These studies suggest that mRNA
oxidation may be an important factor initiating the cascade of neurodegeneration. In our current study we investigate whether RNA oxidation also occur in human ALS patients.

3.3 Materials and methods

ALS tissues

ALS brain and spinal cord tissues were obtained from the Johns Hopkins ALS Brain Bank. Postmortem delays for autopsy were generally less than 12 hr. Pathological confirmation of ALS was made on all specimens by standard histological evaluation of spinal cord and motor cortex, with use of hematoxylin and eosin to evaluation of motor neuron loss, and with myelin stains (Luxol-fast blue) to establish corticospinal tract degeneration.

RNA isolation

RNA isolation was performed according to the manufacturer's protocol. After DNase I (Invitrogen, Carlsbad, CA) treatment, poly (A) + RNAs were selected using the Oligo-tex mRNA Purification Kit (Qiagen). RNA concentration was measured by spectrophotometric ratio A260/A280. Poly (A) + RNAs from 2 individual mice (100μg from each) were combined as an mRNA pool for Immunoprecipitation.

Immunoprecipitation

Poly (A) + RNAs (1.5 μg) were incubated with 1.5 μg of anti-8OHG antibody 15A3 at room temperature for 16 hr. For negative control trials, the primary antibody was omitted or preincubated with 24 ng/μl of 8OHG (Cayman Chemical, Ann Arbor, MI). Immobilized Protein L gel beads (20μl) (Pierce, Rockford, IL) were added and incubated at 4°C for an additional 15 hr. The beads were washed three times with PBS and 0.04% (v/v) NP-40 (Roche Products). Afterward, the following items were added
in the following order: 300 µl of PBS with 0.04% NP-40, 30 µl of 10% (w/v) SDS, and 300 µl of PCI (phenol: chloroform: isoamyl alcohol, 25:24:1). The mixture was incubated at 37°C for 15 min (vortexing every 5 min) and separated to aqueous phase and organic phase by spinning at 14,000 rpm for 5 min. The aqueous layer was collected and mixed with 40 µl of 3 M sodium acetate, pH 5.2, 2 µl of 5 mg/ml glycogen, plus 1 ml of 95% (v/v) ethanol. The sample was frozen at -80°C for 1 hr and centrifuged for 20 min. The pellet was washed with 75% ethanol and air-dried. It was resuspended in 14 µl of DEPC-treated H₂O.

**cDNA synthesis and Southern blotting.**

Immunoprecipitated mRNAs were reversely transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Roche Products). For 15 µl of reaction mixture, 7 µl of immunoprecipitate mRNAs and 1µl (0.75 µg) of oligo-(dT)₂₄-T7 primer were mixed and incubated at 70°C for 10 min. After 2 min on ice, the master mix contained 7 µl of 5x first-strand buffer (Roche Products), 0.5 mM 2'-deoxynucleoside 5'-triphosphates [deoxy (d)-ATP, dCTP and dGTP], 0.13 mM 2'-deoxythymidine-5'-triphosphate, 0.03 mM digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (Roche Products), 2.5 U of RNase Inhibitor (Invitrogen), and 10 U of AMV reverse transcriptase (Roche Products). The mixture was incubated at 42°C for 90 min. Two microliters from 15 µl of digoxigenin-labeled cDNAs were used for additional detection by the Southern blotting method to compare the difference of cDNA quantities between SOD1^{G93A} transgenic and non-transgenic cases. cDNAs were resolved in 1% agarose gel and then transferred electrophoretically to a positively charged nylon membrane (Roche Products) using the Trans-Blot SD semidry transfer system (Bio-Rad, Hercules, CA) according to the
directions of the manufacturer. Digoxigenin labeled on cDNAs was detected with a Digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Roche Products).

Microarray hybridization

Preparation of cRNA was performed according to the Two-cycle cRNA amplification protocol provided by Affymetrix. The immunoprecipitated oxidized mRNA was converted to cDNA using AMV reverse transcriptase and an GeneChip T7-oligo(dT) primer 5’-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24-3’ (Affymetrix, Santa Clara, CA). For the first-cycle IVT amplification of cRNA, MEGAscript T7 kit (Ambion, Inc.) was used. The cRNA is purified and cleanup by using RNeasy mini kit (Qiagen Inc., Valencia, CA, USA). Each cRNA sample was synthesized from 2 independent biological samples. Biotin-labelled cRNA was synthesized from second-cycle cDNA using an IVT Labelling Kit (Affymetrix, Santa Clara, CA). The yield of the in vitro transcription reaction was determined by product absorbance at 260 nm measured by NanoDrop ND-1000 (NanoDrop Technologies, Inc., Montchanin, DE), size of cRNA probes was evaluated by using RNA 6000 Nano LabChip Kit (Agilent, Palo Alto, CA, USA). Following labelling, samples were run experion to examine the quality (Affymetrix, Santa Clara, CA). Fragmented cRNA (15 µg) was used for hybridization to GeneChip Huamn Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA).

3.4 Result

3.4.1 mRNA isolated from ALS affected areas are oxidized

We previously developed an immunoprecipitation procedure using the monoclonal antibody 15A3, which recognizes 8OHG in the oxidized RNA, to isolate oxidized RNA (Shan et al., 2003). We used this established procedure to
investigate whether mRNAs are oxidized in ALS postmortem tissues. The following postmortem tissues were examined: 11 SALS motor cortices, 10 SALS lumbar spinal cords, 2 FALS motor cortices, 5 SALS cerebellums, 5 age-matched normal motor cortices, and 1 age-matched normal lumbar spinal cord. Poly(A)$^+$ mRNAs were isolated from each tissues, and oxidized mRNAs were separated from non-oxidized mRNAs by immunoprecipitation with the 15A3 antibody. The isolated oxidized mRNAs were then reversely transcribed to cDNAs. DIG labeled-dUTPs were incorporated into cDNAs, so as to facilitate analysis by Southern blotting. Among the SALS motor cortex samples, three samples showed a strong signal on the Southern blot analysis such as SALS1 (Fig 3.1A, lane 3), while four samples showed only a moderate signal such as SALS2 (lane 4), and four samples showed a weak signal such as SALS3 (lane 5). Two FALS motor cortex samples showed a moderate signal (lane 6 and 7). Among the SALS spinal cord samples, four samples showed moderate signal such as SALS2 (lane 8), and six samples showed very weak signal such as SALS1 (lane 9). There is no or very weak signals for ALS cerebellum (lane 10), normal motor cortex (lane 11) or normal spinal cord (lane 12). A no-antibody control (lane 1) and an 8OHG-blocked antibody control (lane 2) were also carried out to confirm that the precipitated mRNAs were in fact oxidized mRNAs. Interestingly, tissues from SALS1 patient showed a strong signal in the motor cortex (lane 1) but only a weak signal in the lumbar spinal cord (lane 9); however, tissues from patient SALS2 showed a moderate signal in both the motor cortex (lane 4) and lumbar spinal cord (lane 8). These results indicate that mRNAs are oxidatively damaged to varying degrees in ALS patients. We quantified the relative amount of mRNA oxidation by comparing the signal density of oxidized mRNA to different dilutions of
non-oxidized mRNA via southern blot analysis. Furthermore, we quantified the relative amount of mRNA oxidation by comparing the signal density of oxidized mRNA to different dilutions of non-oxidized mRNA via Southern blot analysis (Fig.3.1B). Quantitative analysis revealed that about 6-10% of the mRNAs were oxidized in those ALS tissues showing relatively strong signal on the Figure 1A Southern blot such as SALS1 motor cortex (Fig.3.1B), and about 3-5% of the mRNAs were oxidized in those ALS tissues showing moderate signal.
Figure 3.1 mRNAs are oxidatively damaged in ALS affected areas. (A) Southern blot analysis of 15A3-immunoprecipitated oxidized mRNAs. SALS, sporadic ALS; FALS, familial ALS; mctx, motor cortex; sc, spinal cord; cbm, cerebellum; no Ab, no antibody; Ab block, antibody block. mRNAs are oxidized to varying degrees in ALS patients. (B) Quantitative analysis revealed that about 3-10% of the mRNAs were oxidized in ALS affected areas. An example of analysis of SALS1 patient is shown.
3.4.2 Some mRNA species are more susceptible to oxidation damage

To identify oxidized mRNA species, we performed DNA microarray using Affymetrix GeneChip Human Genome U133 plus 2.0. This array can analyze ~47,000 transcripts and variants. Six arrays were performed: 3 arrays for ALS lumbar spinal cord and 3 for ALS motor cortex. In each array, oxidized mRNAs were prepared from one ALS patient motor cortex or spinal cord lumbar region, so three patients were analyzed for each. The array results showed that between 5 and 9% of the probes of the arrayed on the GeneChip were detected as present in these six arrays. The detected probes and their relative signal intensities were similar among the three arrays for each motor cortex and spinal cord. A total of 4,089 and 2,541 mRNA species were identified as present in the oxidized mRNAs pool for motor cortex and spinal cord, respectively. These oxidized mRNA species and their relative signal intensities on the chips were similar among the three arrays.

The signal intensities of probes on the Genechips were from ~600 to 12,000. Table 3.1 shows numbers of mRNA species in different signal intensity ranges. We considered that those mRNA species whose signal intensity was over 3,000 were highly oxidized. We then used the computer program Onto-Express (http://vortex.cs.wayne.edu/projects.htm#Onto-Express) to analyze these highly oxidized mRNA species (~525 species) and grouped them according to molecular function. The analysis showed that these mRNA species encoded for proteins that are related to protein synthesis, cytoskeleton, transcription, mitochondrial electron transport chain, oxidoreductase, apoptosis, protein folding, protein degradation pathway, cell
cycle, signal transduction, and structural component of myelin basic protein, etc. (Table 3.2).

Comparison between ALS motor cortex and lumbar spinal cord array showed very few differences among highly oxidized mRNA species. There are only some signal intensity differences in some oxidized mRNA species, which might be due to differential gene expression in motor cortex and in lumbar spinal cord, as summarized in Table 3.3.
<table>
<thead>
<tr>
<th>Signal Intensity</th>
<th>ALS motor cortex microarray</th>
<th>ALS spinal cord microarray</th>
<th>SOD1(G93A) spinal cord microarray</th>
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<tbody>
<tr>
<td>over 5000</td>
<td>215</td>
<td>128</td>
<td>60</td>
</tr>
<tr>
<td>3000~5000</td>
<td>366</td>
<td>186</td>
<td>141</td>
</tr>
<tr>
<td>2000~3000</td>
<td>515</td>
<td>256</td>
<td>157</td>
</tr>
<tr>
<td>1000~2000</td>
<td>1478</td>
<td>819</td>
<td>648</td>
</tr>
<tr>
<td>below 1000</td>
<td>1515</td>
<td>1160</td>
<td>2403</td>
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<tr>
<td>Total</td>
<td>4089</td>
<td>2541</td>
<td>3409</td>
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**Table 3.1:** Numbers of oxidized mRNA species in different signal intensity ranges.
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<th>Gene accession No.</th>
<th>Gene title</th>
<th>Intensity index</th>
<th>SD</th>
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<td>AL515273</td>
<td>eukaryotic translation elongation factor 1 alpha 1</td>
<td>8.2 ± 0.58</td>
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<tr>
<td>BF246436</td>
<td>eukaryotic translation initiation factor 1</td>
<td>6.3 ± 0.93</td>
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<tr>
<td>NM_005875</td>
<td>eukaryotic translation initiation factor 1B</td>
<td>3.2 ± 0.62</td>
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<td>NM_001418</td>
<td>eukaryotic translation initiation factor 4 gamma, 2</td>
<td>4.8 ± 0.2</td>
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<td>NM_001967</td>
<td>eukaryotic translation initiation factor 4A, isoform 2</td>
<td>6.5 ± 1.25</td>
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<tr>
<td>AK026933</td>
<td>eukaryotic translation initiation factor 5</td>
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<td>AI814295</td>
<td>G elongation factor, mitochondrial</td>
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<td>NM_004539</td>
<td>asparaginyl-tRNA synthetase</td>
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<tr>
<td>D84223</td>
<td>leucyl-tRNA synthetase</td>
<td>3.9 ± 0.86</td>
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<td>NM_005548</td>
<td>lysyl-tRNA synthetase</td>
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<td>ribosomal protein S2</td>
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<td>ribosomal protein S20</td>
<td>10 ± 1.09</td>
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<td>NM_001024</td>
<td>ribosomal protein S21</td>
<td>2.8 ± 0.75</td>
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Continued

**Table 3.2:** List of highly oxidized mRNA species in the ALS motor cortex
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<td>AA888388</td>
<td>ribosomal protein S25</td>
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<td>NM_001030</td>
<td>ribosomal protein S27</td>
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<td>NM_002954</td>
<td>ribosomal protein S27a</td>
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<td>NM_001032</td>
<td>ribosomal protein S29</td>
<td>3 ± 0.22 *</td>
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<td>U14990</td>
<td>ribosomal protein S3</td>
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<td>ribosomal protein S4, X-linked</td>
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<td>ribosomal protein S6</td>
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<td>AI970731</td>
<td>ribosomal protein S7</td>
<td>6.1 ± 0.29 *</td>
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</table>
**cytoskeleton**   |                                                 |                 |       |
| NM_021103         | thymosin, beta 10                               | 4.6 ± 0.6 *     |       |
| NM_006317         | brain abundant, membrane attached signal protein 1| 4.5 ± 1.16     |       |
| NM_005507         | cofillin 1 (non-muscle)                         | 6.5 ± 0.59 *    |       |
| BF342661          | microtubule-associated protein 2                | 6.4 ± 0.71      |       |
| AA058571          | abl interactor 2                                | 3.3 ± 0.37      |       |
| NM_003633         | ectodermal-neural cortex (with BTB-like domain) | 3.6 ± 1.02      |       |
| NM_001007         | tubulin folding cofactor A                      | 3.3 ± 0.76      |       |
| NM_001069         | tubulin, beta 2A                                | 5.9 ± 0.82      |       |
| AF141347          | tubulin, alpha 1a                               | 12 ± 1.1        |       |
| BC005946          | tubulin, alpha 1c                               | 3.3 ± 0.61      |       |
| BC004912          | dystonin                                        | 3.8 ± 0.44      |       |
| AB029290          | microtubule-actin crosslinking factor 1         | 5.8 ± 1.21      |       |
| BE965029          | microtubule associated monoxygenase             | 4.1 ± 0.43      |       |
| AI888150          | protein phosphatase 1, regulatory (inhibitor) subunit 9A | 3.9 ± 0.39 |       |
| NM_020987         | ankyrin 3, node of Ranvier (ankyrin G)          | 5.9 ± 1.29      |       |
| BE545756          | adducin 3 (gamma)                               | 3.9 ± 0.55      |       |
| AW190090          | actin, gamma 1                                  | 7.1 ± 1.05      |       |
| AK025873          | actin, beta                                     | 4.9 ± 0.45      |       |
| NM_002628         | profilin 2                                      | 3.8 ± 0.76 *    |       |
| NM_002055         | glial fibrillary acidic protein                 | 6.6 ± 1.14      |       |
| BF055311          | neurofilament, light polypeptide 68kDa          | 7.3 ± 1.99 *    |       |
| NM_005382         | neurofilament, medium polypeptide 150kDa       | 3.6 ± 1.19 *    |       |
| X15306            | neurofilament, heavy polypeptide 200kDa        | 3.8 ± 1.67 *    |       |
| AK054976          | histidine triad nucleotide binding protein 1    | 4.8 ± 0.58      |       |
**microtubule motor activity** |                                  |                 |       |
| BG110975          | dynein, cytoplasmic 1, light intermediate chain 2| 9.2 ± 0.4       |       |
| NM_003746         | dynein, light chain, LC8-type 1                 | 4.4 ± 0.97 *    |       |

Continued
Table 3.2 continued

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<th>Gene accession No.</th>
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<td>NM_006520</td>
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<td>AB051495</td>
<td>kinesin family member 21A</td>
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<td>BF939474</td>
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<td>microfilament motor activity</td>
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<td>myosin VA (heavy chain 12, myoxin)</td>
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**induction of apoptosis**

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**regulation of apoptosis**

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**serine-type endopeptidase inhibitor activity**

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**hydrolase activity**

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**signal transduction**

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Note: Intensity index equals to the average of three arrays divided by 1000. SD, standard deviations among three arrays.
* The mRNA species is also highly oxidized in human ALS array;
** Many subunits of electron transport chain are also highly oxidized in SOD1 (G93A) transgenic mice, but they may have a different name.
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Table 3.3 Comparison of oxidized mRNA species between ALS motor cortex and spinal cord
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Table 3.3 continued

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Continued
Table 3.3 continued

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Note: “M.C.”, the signal intensity of motor cortex divided by 1000. “S.C.”, the signal intensity of spinal cord divided by 1000. “M.C.-S.C.”, difference of signal intensity between “M.C.” and “S.C.”, then divided by 1000.
Comparing the profiles of highly oxidized mRNA species in human ALS motor cortex to 60-day SOD1 (G93A) spinal cord, we found that the profile of oxidized mRNA species of human ALS motor cortex covers the majority of the oxidized mRNA species that were evident in SOD1 (G93A) transgenic mice, including mRNA encoding for protein synthesis, cytoskeleton, mitochondrial electron transport chain, protein folding and protein degradation, etc. More mRNA species encoding protein involved in signal transduction, hydrolase activity, oxidoreductase activity calcium binding, and zinc ion binding, etc, were highly oxidized. In addition, more mRNA species showed highly oxidized in human ALS but not in SOD1 (G93A) mouse array, including mRNA species encoding protein related to transcription, apoptosis, cell cycle regulation, immune response, etc. Table 3.2 labeled the highly oxidized mRNA species that also showed highly oxidized in mouse array.

Comparing the profiles of oxidized mRNA species in ALS motor cortex and AD frontal cortex, surprisingly, it appears quite similar, except some differences in the signal intensity of some mRNA species, as summarized in Table 3.3. The signal intensity difference may highly relate to differential gene expression in between motor cortex and frontal cortex. It could be also due to alteration of gene expression profile during the disease process of AD or ALS. One major difference is that in ALS motor cortex, the signal intensity of the mRNA species related to oligodendrocytes are high, such as myelin basic protein, proteolipid protein, claudin11 suggesting RNA oxidation involvement in different cell types in these two neurodegenerative disease.
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**Table 3.4:** Comparison of oxidized mRNA species in ALS motor cortex and AD frontal cortex

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Table 3.4 continued

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<td>NM_021136</td>
<td>reticulon 1</td>
<td>10.0±0.4</td>
<td>7.0±0.6</td>
<td>3</td>
</tr>
<tr>
<td>NM_014282</td>
<td>hyaluronan binding protein 4</td>
<td>1.1±0.1</td>
<td>1.9±1.7</td>
<td>-1</td>
</tr>
<tr>
<td>NM_006574</td>
<td>chondroitin sulfate proteoglycan 5 (neuroglycan C)</td>
<td>2.6±0.1</td>
<td>3.8±0.5</td>
<td>-1</td>
</tr>
</tbody>
</table>

Note: “M.C.”, the signal intensity of ALS motor cortex divided by 1000, “F.C.”, the signal intensity of AD frontal cortex divided by 1000, “M.C.-F.C.”, the difference of signal intensity between “M.C.” and “S.C.”, then divided by 1000.
3.5 Discussion

In the ALS postmortem tissues study (Fig. 3.1), we found that mRNAs are oxidatively damaged to varying degrees in ALS patients. In general, ALS motor cortex tissues contain more oxidized mRNA than ALS spinal cord tissues. Quantitative analysis revealed that about 3 to 10% of the mRNAs were oxidized in ALS tissues. Consistently, in SOD1 (G93A) mice (Chapter 2, Fig. 2.3), we observed more oxidized mRNA in the pre-symptomatic stage (~30%) than in the end stage (~10%). Furthermore, our previous study in AD postmortem tissues indicates that less oxidized mRNAs are present in the more advanced patients and more affected areas. It is possible that significant numbers of neurons are degenerated so that a lower amount of oxidized mRNAs is present. These results suggest that RNA oxidation may be a common early event preceding motor neuron degeneration in ALS patients.

Interpretation of human postmortem tissue pathological changes is difficult, since postmortem tissue represents the very end stage of the neurological disease and reflects the cells that remain but not necessarily those that are at risk. That is the reason we did the pre-symptomatic stage SOD1 (G93A) transgenic mouse array at the very beginning, however, we were curious about the profile of oxidized mRNA species in human ALS, we want to examine whether those oxidized mRNA species in 60-day SOD1 (G93A) transgenic mice are also oxidized in human, and find some clues to prove that the pathological changes found in 60-day SOD1 (G93A) transgenic mice also happens in human ALS. The analysis of the human ALS motor cortex array showed many more groups of mRNA have been oxidized when comparing it to pre-symptomatic
stage SOD1 (G93A) array. Differentially oxidized mRNAs have been categorized based on their biological process and include mRNAs corresponding proteins involved in transcription, apoptosis, immune response, cell cycle, hydrolase activity etc. On the other hand, more oxidized mRNA species were identified in the categories found in mice, like calcium ion binding, zinc ion binding, and signal transduction, etc. This difference of oxidized mRNA profile between the pre-symptomatic stage mouse array and the end-stage human ALS array might be due to: (1) the human array can analyze 49,000 transcripts, while the mouse array only can analyze 37,000 transcripts. (2) The human array has more known genes than mouse array. (3) Altered transcriptional profile at different disease stage. At the end-stage of the disease, the motor neurons are dying, microglia is activated, and the glial cells are proliferating. It is a decompensate state, and those genes which are upregulated in motor neurons at early disease stage try to protect them from degeneration begin to down regulate. (4) Cell population of RNA oxidation. Immunoreactivity of oxidized RNA of lumbar spinal cord sections of different ages of SOD1 (G93A) mice with 15A3 shows that at pre-symptomatic stage (60 days) oxidized mRNA mainly located within neurons and oligodendrocytes. At end stage the dying motor neurons showed less RNA oxidation, while the glial cells began to show increased RNA oxidation. This is consistent with our findings that some marker mRNAs for glial cells such as excitatory amino acid transporter 2 (EAAT2) and glial fibrillary acidic protein (GFAP) showed highly oxidized in end-stage ALS motor cortex and spinal cord. In addition, at pre-symptomatic stage, increased free radicals were mainly localized in the neurons, and the relative contact cytoskeleton structures of motor neuron protect some mRNA species without being oxidized. As the disease progress, the free radical
level is increased; the overall transcription was inhibited in motor neurons. At end stage motor neuron was dying and the primary structure of motor neurons were disrupt, results in some mRNA species showed oxidized at end stage of the disease but absent at early stage, such as Microtubule-associated protein 2 (MAP-2), and excitatory amino acid transporter 1(EAAT1). Thus, the oxidized mRNA species identified at end stage may belong to the combination of any following cell type: dying motor neurons, oligodendrocytes, other types of neurons, glial cells, or microglia. Interestingly, even at such a devastating stage, mRNA oxidation is still selectively oxidized.

In the human ALS array, there are more groups of oxidized mRNA showed up, including encoding protein related to apoptosis, cell cycle, immune response, and transcription etc. These oxidized mRNA oxidized species may not contribute to the disease, since at the end stage of the disease the motor neurons are died or dying. However, oxidation of these groups of mRNA species may accelerate the process of motor neuron death. The following discussion is about the relationship of individual mRNA species and their role in neurodegeneration.

**Apoptosis**

In ALS, apoptosis plays an important role in the motor neuron death. The following mRNA species are oxidized in ALS motor cortices and spinal cord, and the disturbances of these factors may accelerate the process of motor neuron death.

The altered expression level of Bcl-2 family oncoproteins can promote apoptosis. In one ALS transgenic mouse model, overexpression of Bcl-2 protein can delay the disease onset, prolonging the lifespan. Bcl-2 family genes regulate apoptosis by a variety of different mechanisms, including direct activation of caspases, or control of
the mitochondrial potential or cytochrome c release by formation of channels in the mitochondrial membrane. **Bcl-2-associated transcription factor 1**, a transcriptional repressor, interacts with several proteins of the Bcl-2 family. It is reported that sustained overexpression of this protein in HeLa cells induced apoptosis, which was suppressed by co-expression of Bcl-2 proteins (G. M. Kasof et al., 1999). **Bcl-2/adenovirus E1B 19kDa interacting protein 3**, **BNIP3**, encodes a dimeric mitochondrial protein, which can induce apoptosis, even when co-expressed with Bcl-2 (Ray et al., 2000).

**Clusterin**, also termed apolipoprotein J is a glycoprotein that has been implicated in cell death and remodeling in different tissues. In the brain and spinal cord, clusterin mRNA is present in both neurons and glia. However, in a variety of disease states including AD, seizures and hypoxia-ischemia, clusterin mRNA levels are predominantly upregulated in astrocytes (P. L. McGeer et al., 1992; S. S. Schreiber et al., 1993; S. E. Jones and C. Jomary, 2002). Han et al. (2001) generated a clusterin knock out mice which had 50% less brain injury following neonatal hypoxia-ischemia, which indicate that clusterin can exacerbate neuronal injury (Han et al., 2001).

**Reticulon 4 (Nogo)** is a potent neurite outgrowth inhibitor which is involved in denervation in an ALS transgenic animal model (Jokic et al., 2006). It is reported that the expression level of reticulon 4 in ALS muscles is correlated with the severity of the disease symptoms and that it promotes retraction of the motor nerve presynapse and contributes to the axonal and motor neuron degeneration (Jokic et al., 2006). Upregulation of Nogo-A in diseased human muscle biopsies is not unique to ALS, as it is also present in other neuromuscular disease (Wojcik et al., 2006). Increased expression of reticulon 4 was reported in schizophrenia and hippocampal neurons of
patients with temporal lobe epilepsy (G. Novak et al., 2002; C. E. Bandtlow et al., 2004).

**NGFR-associated protein 1**, also called NADE, a p75NTR-associated cell death executor, which interacts with p75NTR death domain to induced NGF dependent cell death. p75(NTR) has been implicated in motor neuron death occurring in mutant Cu-Zn superoxide dismutase (SOD1) transgenic mice (O. Bai et al., 2002; B. J. Turner et al., 2003). Recently one study has shown that in SOD1 (G93A) motor neuron culture, p75NTR-mediated apoptosis induced by NGF involved ceramide-dependent increase in mitochondrial superoxide production, and cytochrome c release. The increased susceptibility of SOD1 (G93A) motor neurons to NGF was associated with decreased nuclear factor erythroid 2-related factor 2 (Nrf2) expressions and downregulation of the enzymes involved in glutathione biosynthesis. This apoptotic pathway is enhanced by the overexpression of mutant SOD1 and critically modulated by Nrf2 activity (M. R. Vargas et al., 2006).

**Forkhead box O3** belongs to the forkhead family of transcription factors; this gene might function as a trigger for apoptosis through expression of genes necessary for cell death (M. L. Asselin-Labat et al., 2004). Decreased expression of ROS scavenging enzymes were observed in Foxo3-deficient erythrocytes and such erythrocytes showed increased oxidative damage and a shortened lifespan (Marinkovic et al., 2007).

**Tumor protein, translationally-controlled 1 (TPTC1)**, is ubiquitously expressed; it appears to regulate cell proliferation and survival. TCTP1 can bind to calcium, tubulin, and myeloid cell leukemia 1 protein, and possesses a self-interaction property as well (Y. Gachet et al., 1999; T. Yoon et al., 2000; U. A. Bommer et al., 2002; D. Zhang et al., 2002). Overexpression of TCTP1 was observed to stabilize microtubules, and
change cell morphology. TCTP1 levels are usually altered in various stress conditions and extracellular signals, including growth factors, cytokines, starvation, heat shock, heavy metals, and apoptosis signals (A. Xu et al., 1999; C. Bonnet et al., 2000; F. Li et al., 2001; P. Thaw et al., 2001). It suggests that TCTP1 may play a cytoprotective role. TCTP is also involved in cell survival and regulation of apoptosis. In the present study, the results showed that the mRNA species corresponding to TCTP1 was highly oxidized in both spinal cord of SOD1 (G93A) mice and also in ALS patients, suggesting that the cytoprotective function and the calcium binding activity and/ or the regulation of apoptosis of TCTP might be disturbed.

**High mobility group box protein 1 (HMGB1)** is a ubiquitous nuclear protein that exerts numerous cytoplasmic and extracellular functions, including a pro-inflammatory activity, able to induce cytokines expression and activate inflammatory cells. Lo Coco, et al. (2007) investigated whether this protein may play a role in the inflammatory events in ALS, and found high expression levels of HMGB1 in the ventral horn motor neurons, in both non transgenic and pre-symptomatic transgenic SOD1 (G93A) mice (Lo Coco et al., 2007). In the disease stage, HMGB1 is decreased in degenerating motor neurons, and is increased in the nucleus of glial cells, as determined by immunostaining. The altered expression and localization of HMGB1 in the spinal cord may contribute to the progression of inflammatory and neurodegenerative processes.

**Transcription**

Hypoxia-inducible factor-1 (HIF1) is a transcription factor which plays an essential role in maintaining cellular homeostatic responses to hypoxia. HIF activity is regulated by multiple mechanisms including modulation of protein stability,
nuclear translocation, transactivation activity and transcriptional regulation of mRNA expression level in response to physiological or pathological oxygen concentrations. HIF-1 activates transcription of various genes, including those encoding proteins involved in angiogenesis, such as vascular endothelial growth factor (VEGF), in energy metabolism, such as some glycolytic enzymes involved in anaerobic ATP biosynthesis, iron homeostasis and cell survival. HIF-1 induction has mostly been implicated in neuroprotection in AD (Semenza et al., 2000). Activation of HIF-1α could prevent Aβ1–42-induced neurotoxicity, which suggests a neuroprotective role of HIF-1α in AD (T. Soucek et al., 2003). Oosthuyse et al. (2001) generated mice by deletion of the hypoxia-response element in the Vegf promoter, with reduced hypoxia-induced Vegf expression in the spinal cord, and they found that these mice develop an adult-onset, progressive motor neuron degeneration ALS-like disease. The neurodegeneration seemed to be related to either reduced neural vascular perfusion or to insufficient VEGF–dependent neuroprotection (B. Oosthuyse et al., 2001). Another study showed that all ALS patients have a reduced VEGF protein level in the serum (D. Lambrechts et al., 2003). These data indicate reduced VEGF expression is a modulator of ALS pathology in the human situation. Thus, dysfunction of HIF-system would lead to reduced induction of neurotrophic adaptive mechanisms, and facilitate cell death.

**Immune response**

*β2-microglobulin* is a component of MHC class I complex, which plays an important role in antigen presentation and immunoglobulin transport (Tysoe-Calnon et al., 1991). In the nervous system, β2-microglobulin might be involved in the synaptic plasticity of neurons (R. A. Corriveau et al., 1998; G. S. Huh et al., 2000; A. L.
Oliveira et al., 2004). Upregulation of β2-microglobulin and MHC-I mRNA expression has been reported in aged motor neurons (Edstrom et al., 2004). Other than the physiological role of MHC class I in neurons, constitutive β2-microglobulin expression is related to selective vulnerability, in groups of neurons which are often affected by neurodegenerative diseases such as motor cortex and spinal cord neurons in ALS, and substantia nigra pars compacta neurons in PD. In addition, specific motoneurons and dopaminergic neurons which are relatively spared in ALS and PD, such as the ocular motor neuron, exhibit lower β2-microglobulin mRNA expression.

**Others**

**tRNA synthetase** In the category of protein biosynthesis, several tRNA synthetase mRNA showed oxidation. Low levels of defective transfer RNAs (tRNAs) can lead to an intracellular accumulation of misfolded proteins in neurons, which might contribute to neurodegeneration. A missense mutation in alanyl-tRNA synthetase, results in cerebellar Purkinje cell loss, leading to ataxia (Lee et al., 2006). Charcot-Marie-Tooth (CMT) disease, an inheritable peripheral neuropathy caused by demyelination or axonal degeneration, has at least 10 mutant alleles in the gene encoding for glycyl-tRNA synthetase. Another group identified heterozygous missense mutations tyrosyl-tRNA synthetase (YARS) in three unrelated families (A. Jordanova et al., 2006). Kunst, et al. (1997) reported that lysyl-tRNA synthetase interacts with mutant SOD1 but not with wild-type SOD1 (Kunst et al., 1997). This evidence suggest that mutation in some tRNA synthetases caused by the corresponding mRNA oxidation might contribute to neurodegeneration.
Glutamate-mediated excitotoxicity plays a major role in various neurodegenerative diseases. EAAT2 is one of the glutamate reuptake transporter proteins localized in glial cells, and is essential for the removal and termination of action of the excitatory neurotransmitter glutamate from the synaptic cleft. Northern blotting revealed that the quantity and size of EAAT2 mRNA are normal in the ALS motor cortex, even in patients with a large loss of EAAT2 protein (L. A. Bristol and J. D. Rothstein, 1996; C. B. Kunst et al., 1997; C. L. Lin et al., 1998). In the current study, EAAT2 mRNA has not been oxidized in the 2-month mouse spinal cord, but is oxidized in the ALS post mortem motor cortex and spinal cord, suggesting mRNA oxidation of EAAT2 is related to astrogliosis at the end stage of disease. Oxidized EAAT2 mRNA may enhance the loss of EAAT2 protein, leading to accumulation of extracellular glutamate, and resulting in excitotoxicity-induced neuronal death.

*Quaking protein*, a RNA binding protein, regulates oligodendrocyte differentiation and maturation, thus, is essential for myelination. The quaking mutant mouse exhibits severe dysmyelination of the central nervous system (Hardy, 1998). Quaking mutant mice develop some pathological changes similar to those observed in schizophrenia. In addition, quaking mRNA expression level in the brain was found to be decreased in the brains of schizophrenia patients (Haroutunian et al., 2006).

*Selenoprotein P*, plasma, 1 is an extracellular glycoprotein that has several isoforms, and is the only selenoprotein known to contain multiple selenocysteine residues. It has been implicated as an antioxidant defense in the extracellular space. Selenium deficiency is associated with nutritional muscular dystrophies in several species; it
might be of interest to evaluate SEPP1 in that form of muscular dystrophy (P. Lindberg and M. Siren, 1963; H. F. Hintz and D. E. Hogue, 1964). Significant elevation of selenium was found in the spinal cords of motor neuron disease patients (Ince et al., 1994). Other selenoproteins include glutathione peroxidase-1, thioredoxin reductase, glutathione peroxidase-2, glutathione peroxidase-3, thyroxine deiodinase type 1, and mitochondrial capsule selenoprotein. Dysfunction of selenoprotein may contribute to oxidative damage.

Comparison of human ALS array to AD array suggests that many of oxidized mRNA species are general to these neurodegenerative diseases. Thus some common features of the neurodegenerative disease like protein aggregation, mitochondria dysfunction, might be related to mRNA oxidation. On the other hand, the similarity of oxidized mRNA oxidation may not contribute to the selectively vulnerability of motor neurons of spinal cord and motor cortex in ALS or neurons of hippocampus in AD.

In summary, we found that different amounts of mRNA had been oxidized in the motor cortex and lumbar spinal cord of both sporadic and familial ALS patients, but not in normal patients or in ALS disease spared tissues. Further identification of the oxidized mRNA species revealed that the human ALS array covers the majority of oxidized mRNA species that showed up in 60-day SOD1 (G93A) transgenic mouse array, and showed more categories of oxidized mRNAs corresponding proteins involved in apoptosis, cell cycle, immune response, transcription, hydrolase activity related. Surprisingly, comparison profile of oxidized mRNA species in AD frontal cortex arrays to ALS motor cortex arrays showed that the oxidized mRNA species in these two neurodegenerative diseases at end stage are quite similar. These results indicate
that mRNA oxidation is a general early pathological alteration in ALS pathogenesis or even in other neurodegenerative diseases, oxidized mRNAs at early stage may participate the initiation of motor neuron degeneration, and at the end stage in which motor neurons are dying, oxidized mRNA may or may not accelerate the neurodegeneration process.
4.1 Abstract

Transgenic mice expressing mutant Cu^{2+}/Zn^{2+} superoxide dismutase SOD1 (G93A) develop similar clinical and pathological phenotypes to amyotrophic lateral sclerosis (ALS) patients. Here, we utilize representational difference analysis to identify the transcripts which are upregulated in the pre-symptomatic stage of SOD1 (G93A) mice. Unexpectedly, three predominant clones were 18S or 28S ribosomal RNA (rRNA) segments. One of these clones corresponded to a capped and polyadenylated transcript containing a large portion of 18S rRNA, named MSUR1 (mutant SOD1-upregulated RNA 1). *In vitro* expression experiments show that MSUR1 is able to rescue SOD1 (G93A)-mediated cell death. Expression of MSUR1 significantly reduces SOD1 (G93A)-induced free radical levels and oxidative damage. Further, MSUR1 can reduce hydrogen peroxide-mediated cytotoxicity. MSUR1 does not encode a protein, suggesting its role as a functional non-coding RNA. It is widely expressed in various tissues. Searching the database of GenBank revealed that a large number of expressed sequence tag (EST) clones contain large portions of rRNA sequence, potentially indicating a heretofore overlooked class of mRNAs with functional significance.
4.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that is clinically characterized by muscle wasting, weakness, and spasticity, reflecting the relatively selective degeneration of motor neurons in the spinal cord, motor cortex and brainstem, which typically results in mortality within 2-5 years after the onset of disease (Mulder et al., 1986). The majority of cases have no genetic component. Approximately 5% of ALS cases are familial (FALS), with an autosomal dominant inheritance pattern. About 15-25% of the cases of FALS are linked to mutation in the gene encoding the antioxidant enzyme Cu\(^{2+}\)/Zn\(^{2+}\) superoxide dismutase (SOD1). The causes for most cases of ALS are unknown. It is believed that multiple factors underlie the disease mechanism (Rosen et al., 1993; Bruijn et al., 2004; Boillee et al., 2006).

Overexpression of some FALS-linked mutant SOD1 proteins (i.e. G93A, G37R and G85R) in transgenic mice results in the development of a neurological disorder that resembles ALS (Gurney, 1994; Ripps et al., 1995; Wong et al., 1995; Bruijn and Cleveland, 1996). Mutant SOD1 causes motor neuron degeneration by acquiring a toxic gain of function property, rather than by the loss of enzymatic activity (Borchelt et al., 1994; Reaume et al., 1996; Bruijn et al., 1998). The molecular mechanisms underlying mutant SOD1-linked FALS remain unclear. Many studies have shown that the mutant SOD1 toxicity to motor neurons is non-cell autonomous, meaning that mutant damage is required within both motor neurons and non-neuronal cells in order to fully represent the ALS phenotype (Gong et al., 2000; Pramatarova et al., 2001; Clement et al., 2003; Beers et al., 2006; Boillee et al., 2006).
The transgenic mouse expressing mutant SOD1(G93A) is a commonly used ALS animal model (Gurney, 1994). These mice show obvious loss of motor neurons and associated motor function at about 3 months of age and eventually die at about 4-5 months of age. There is no motor neuron loss at 2 months of age. The original idea of this study was that there may be factors induced at an early age having protective functions against mutant SOD1(G93A) toxicity. We performed representational difference analysis (RDA) (Hubank and Schatz, 1994), a PCR-based subtraction hybridization method, and identified a novel transcript named MSUR1, which was upregulated in early age (1-2 months old) of SOD1(G93A) mice. The data presented here shows that MSUR1 is a member of a group of polyadenylated transcripts that contain large portions of 18S or 28S rRNA sequence. Importantly, MSUR1 is capable of reducing mutant SOD1 (G93A)-mediated toxicity and cell death.

4.3 Materials and Methods

Mice

SOD1 (G93A) (Strain B6SJL-TgN(SOD1-G93A)1Gur) and wild-type SOD1 (Strain B6SJL-TgN(SOD1)2Gur) as well as nontransgenic (Strain B6SJLF1/J) mice were purchased from The Jackson Laboratory.

Representational difference analysis (RDA)

RDA was conducted with cDNAs derived from poly(A)^+ RNA prepared from the spinal cord of SOD1(G93A) transgenic or nontransgenic sibling mice according to Hubank and Schatz (1994) except that the third and final hybridization was carried out at a tester:driver ratio of 1:100,000 instead of 1:400,000. The transgenic SOD1(G93A)
mouse sample was used as the tester and the nontransgenic mouse sample was used as the driver. We examined three different ages of mice including 4-week-old, 2-month-old and 4-month-old.

**Poly(A)$^+$ RNA isolation**

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. Poly(A)$^+$ RNA was selected from total RNA using the Oligotex mRNA Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s directions. To ensure that all possible non-poly(A)$^+$ RNA were removed, RNA was passed through the selection process twice and washing steps were increased in number from two to three.

**Reverse transcription (RT) and 3′ rapid amplification of cDNA ends (3′ RACE)**

1st strand cDNA was synthesized from poly(A)$^+$ RNA reverse transcribed using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. Polymerase chain reaction (PCR) was performed at a final concentration of 1x PCR buffer/3mM MgCl$_2$/0.3 mM dNTPs/0.4 µM each 5′ and 3′ primers/10% DMSO/2 µl of RT reaction mixture in a total of 50 µl. The mixture was amplified with an MJ Research PTC-200 thermal cycler for 30 cycles. Initial denaturation was at 98°C for 5 min, followed by 20 sec at 75°C during which 2.5 units Taq polymerase (Invitrogen, Carlsbad, CA, USA) were added to each sample. Initial annealing was 2 min at 55°C followed by 40 min at 72°C. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, primer extension at 72°C for 3 min. Final extension was at 72°C for 15 min. Primers used for PCR were as follows: primer B (5′-GGTCTGTGATGCCCTTAGATGTCCG-
3′) corresponding to position +1490 of 18S rRNA gene; primer E (5′-GATGTCGGCTTCTCCTATCATTGAA-3′) corresponding to position +4098 of 28S rRNA gene; primer T7-(dT)$_{24}$ (5′-AAACGACGGCCAGTAATCGACTCATACTATAGGCGC-3′); primer Q1 (5′-GTGAATTGTAATCGACTCATACTATAGGCGC-3′).

To examine non-specific binding of T7-(dT)$_{24}$ to stretches of adenine, RT was performed as described above using total RNA. Primers used were T7-(dT)$_{24}$, Q1, primer A (5′-GGGTGACGGGGAATCGGGTTC-3′) corresponding to position +404 of 18S rRNA gene, and primer C (5′-CAGTCGGTCCTGAGAGATGGGC-3′) corresponding to position +2191 of 28S rRNA gene. PCR conditions were the same as described above.

To examine whether the transcripts corresponding to the identified PCR products (Fig. 1B) contain the RDA fragment regions, the same RT product prepared from poly(A)$^+$ RNA was used. Primers used were primer A, primer +313 (5′-GATCGCACGCCCCCCTGA-3′) corresponding to position +313 of 18S rRNA gene, primer F (5′-GAGCAGTTTTAATGAGGGTGCAGTGTAC-3′) corresponding antisense to the junction between 18S rRNA gene sequence and the 3′ unknown sequence region, and primer G (5′-GGCATTGAGAGGACCAGGCACAGTATC-3′) corresponding antisense to the 28S unknown sequence region. PCR conditions were the same as described above.

5′ RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE)

5′ RLM-RACE was performed using the First Choice RLM-RACE Kit (Ambion, Austin, TX, USA) according to manufacturer’s protocol. Briefly, poly(A)$^+$ RNA was
isolated from mouse cerebral cortex tissue as described above, processed with the kit enzymes to ligate the 5’ RACE adapter to capped, full length mRNA transcripts. 2 step nested PCR was then performed as per above described protocol using kit provided as well as primers +530AS (5′-TTGTTATTTTTTCGTCACTACCTCCCTCCCG-3′) corresponding antisense to position +530 of 18S rRNA gene for step 1 and primer +475AS (5′-CTGCCTTCCTTGGATGTGGTAGCC-3′) corresponding antisense to position +475 of 18S rRNA gene for step 2.

**Northern blot analysis**

500 ng of poly(A)$^+$ selected RNA were fractionated by electrophoresis through a 1.9% formaldehyde/1.2% agarose gel and transferred to positively charged nylon membranes (Roche, Indianapolis, IN, USA) by capillary action overnight in 10x SSC. RNA was fixed to the membrane by UV crosslinking twice and baking at 80°C for 30 min. Antisense RNA probes were labeled with digoxigenin (DIG) -11-UTP (Roche) by *in vitro* transcription using T7 RNA Polymerase (USB, Cleveland, Ohio, USA) for 90 min. at 37°C according to the manufacturer’s protocol. Membrane was prehybridized 4 hr at 65°C and hybridized 16 hr at 65°C. Stringency washing conditions were 2X 5 min at 65°C in 1x SSC, 0.5% SDS, 0.1% Sarkosyl followed by 2X 15 min at 65°C in 0.5x SSC, 0.1% SDS. Additional very high stringency washing conditions were 2X 15 min at 70°C in 0.2x SSC, 0.2% SDS. Antibody incubation and detection followed the manufacturer’s protocol (Roche, Indianapolis, IN, USA). When duplicate blots were required, i.e. multiple tissue blots, individual mRNA samples were prepared in bulk and split to equal loading volumes to ensure equal loading of lanes. Equal loading was confirmed in each case by hybridization to a mouse β-actin probe transcribed from pTRI-Actin-Mouse.
(Ambion, Austin, TX, USA) corresponding to nucleotides 739-989 of GenBank accession no. X03672.

**Transient transfection and Western blot analysis**

HEK293 cells (American Type Culture Collection, Manassas, VA) were transiently transfected with indicated plasmid DNA using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA, USA) as described previously (Guo et al., 2002). Cells were harvested 72 hr after transfection. NSC34 cells (kindly provided by Dr. Neil Cashman, University of Toronto, and Dr. Rugao Liu, University of North Dakota) were transiently transfected with indicated plasmid DNA using electroporation. In brief, after NSC34 cells were incubated with 10 µg plasmid DNA for 10 min at room temperature in 200µl Opti-Mem (Invitrogen, Carlsbad, CA, USA), they were electroporated under 250V, 50ms, 2 pulses by Electro Square Porator ECM 830 (BTX, San Diego, CA). The transfected cells were then incubated at room temperature for 30 min and transferred to petri-dishes. For Western blot analysis, protein extracts were generated from transfected cells, resolved by SDS-PAGE and transferred onto PVDF membranes as described previously (Butchbach et al., 2002). A rabbit anti-SOD1 pAb (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. The immunoreactive bands were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer’s directions.

**RT-PCR analysis**

Total RNA were isolated as described above and first-strand cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using MSUR1-specific primer (5’-
AGCAATTTCAGAGCAGTTTTAATGAGGGTGC-3', corresponding to position +1701 of MSUR1 or 5'-GAGCAGTTTTAATGAGGGTGCAGTGTGTAC-3', corresponding to position +1692 of MSUR1. SOD1-specific primer (5'-CAGCAGTCACATTGCCCARGTCTTCAACATG-3', corresponding to position +349 of mouse SOD1) or β-actin-specific primer (5'-TGTCGAAGAAGGTGTAATAACGCAGC-3', corresponding to position +1223 of mouse β-actin) were used as controls. The following primers were used for PCR: MSUR1 F +1490 (5'-GGTCTGTGATGCCCTTAGATGTCCG-3') and R +1701 (5'-AGCAATTTCAGAGCAGTTTTAATGAGGGTGC-3' or R +1692 GAGCAGTTTTAATGAGGGTGCAGTGTGTAC-3'), SOD1 F +1 (5'-ATGGCGACGAAGGCCGTGTGCGT-3') and R +349 (5'-ATGGCGACGAAGGCCGTGTGCGT-3'), and β-actin F +626 (5'-TGTCAAAGAAGGTGTAATAACGCAGC-3') and R +1223 (5'-CGGGACCTGACAGACTACCTCAT-3'). PCRs were performed in the presence of 3 mM MgCl₂, 0.2 mM dNTP, 0.25 μM primers, and 2 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) in 1x PCR buffer. 25 (for controls) or 35 (for MSUR1) cycles was performed (95°C for 30 s, specific annealing temperature to each set of primers for 45 s, and 72°C for 1 min). PCR products were visualized as single bands on 1% agarose gels stained with ethidium bromide.

**DCF Fluorescence Detection**

The method for fluorescence detection of oxidative stress was slightly modified from the original as described by Wang and Joseph (1999). Cell cultures were grown at least 24 hr before assay. Media were removed and replaced with 1x PBS containing 100 μM...
H$_2$DCFDA. Cultures were placed back into the incubator for 30 min followed by washing with PBS. Cultures were flooded with media containing 400 µM or 700 µM hydrogen peroxide at 37°C for 30 min and then placed in a multiwell fluorescence microplate reader maintained at 37°C. Culture fluorescence was followed for 30 min at an excitation 485 nm and an emission at 530 nm. Data points were taken every 5 min. After the final measurement at 30 min, the percentage of increase was determined using the formula $[\frac{(F_{T30} - F_{T0})}{F_{T0}} \times 100]$, where $F_{T0}$ = fluorescence at 0 min and $F_{T30}$ = fluorescence at 30 min.

**Protein oxidation assay**

Protein extracts were incubated with 10 mM DNPH in 2N HCl at room temperature for 1 hr. One volume of 20% TCA were added to each sample and incubated for 30 min on ice to facilitate the protein precipitation. The precipitated proteins were collected by centrifugation at 4°C with maximum speed for 20 min, washed with cold acetone, air-dried, and then added 1x SDS loading buffer. Western blotting was performed and used anti-dinitrophenyl-KLH antibodies to recognize DNPH binding oxidized protein (Invitrogen, Carlsbad, CA, USA).

**MTT assay**

For the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay, cells were plated at least 48 hr before experiment. Cells were rinsed with 1x PBS and then incubated with 25µg/ml MTT in DMEM (Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C. The reaction was stopped by adding 1 ml of stop solution (0.04 M HCl in isopropanol). Shaking to completely dissolve crystals on the bottom of the plate, the absorbance of violet color was measured in a computer-controlled microplate reader.
(Model 3550, Bio-Rad Laboratories, Richmond, CA, USA) at wavelengths of 570 nm.

**Deletion constructs**

For the deletion #1, pcDNA3/MSUR1 was digested with *BamHI* and then religated to delete +1 to +591 resulting in missing ORF1-3. For the deletion #2, pcDNA3/MSUR1 was digested with *ApaI* and then religated to delete +1 to +794 resulting in missing ORF1-3 and part of ORF4.

**In vitro translation**

MSUR1, deletion #1, SMN and luciferase cDNAs in pcDNA3 vector were used as DNA templates for TNT Quick Coupled Transcription/Translation Systems in the presence of T7 RNA polymerase and [³⁵S] methionine according to manufacturer protocols (Promega, Madison, WI, USA). The reactions were carried out at 30°C for 90 min. The synthesized protein products were electrophoretically resolved through a 7 M urea 18% SDS-PAGE Bis-Tris gel, which was able to resolve peptides of less than 2.5 kDa; the gel was then dried and exposed to autoradiography film for 24 hr.

**Site-directed mutagenesis**

Quikchange® Site-Directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used according to the manufacturer's protocol. The following single point mutations on the MSUR1 ORF4 and MSUR1 ORF5 were introduced via site-directed mutagenesis: T770A and T919A, respectively. Mutagenesis was performed with pcDNA3/MSUR1 as a template using the primers T770A sense primer (5’-CGCCCCCCTCGATGCTCGATGCTGAGTGT-3’), T770A antisense primer (3’-GCGGGGGAGCTACGAGATTGCAGACTCACA-5’), T919A sense primer (5’-
CGGA ACTGAGGCCATGATTAGAGGACGG-3’), and T919A antisense primer (3’-GCCTTGACTCCGGA CTAAATCTCCCTGCC-5’). The mutations were confirmed by sequencing.

4.4 Results

4.4.1 Identified RDA clones correspond to the capped and polyadenylated transcripts containing large portions of rRNA sequence

RDA was performed using cDNAs derived from poly(A)+ RNA prepared from the spinal cords of SOD1(G93A) transgenic or nontransgenic sibling mice. The transgenic SOD1 (G93A) mouse sample was used as the tester and the nontransgenic littermate sample was used as the driver. The products resulting from RDA were expected to be upregulated or newly induced mRNA in the spinal cord of SOD1 (G93A) transgenic mice. We examined three different ages of mice including 1-month-old, 2-month-old and 4-month-old. Among the identified clones, three caught our attention because they were the predominant clones found in the pre-symptomatic stage (1 and 2 months of age), and they corresponded to two related genes. Clone #1 corresponded to 18S rRNA (hereafter referred to as clone 18S), and clones #2 and #3 corresponded to two different parts of 28S rRNA (hereafter referred to as clone 28S-1 and clone 28S-2, respectively) as shown in Figure 4.1A.

We first tested whether these clones were simply artifacts resulting from the oligo(dT) primer nonspecifically binding to the short stretches of adenine present in contaminating 18S or 28S rRNA during reverse transcription (RT) reaction. The results showed that these clones were not artifacts generated from RT of rRNA. We then
examined the following two possibilities: (1) some portion of 18S or 28S rRNA may have poly(A) tails and this subset is upregulated in SOD1(G93A) transgenic mouse, and/or (2) the upregulated transcripts may contain portions of 18S or 28S rRNA sequence. RT-PCRs were performed using poly(A)$^+$ RNA as the template and oligo(dT)$_{24}$-T$^7$ as the primer for RT. Primers used for PCR are indicated in Figure 4.1A: primer B and Q1 for 18S and primer E and Q1 for 28S. If there were rRNAs with poly(A) tails present in SOD1 mouse, we expected to obtain PCR products having complete 3′ ends from the PCR primers (primer B or E) sites followed by poly(A) tail. We did obtain PCR products for both 18S and 28S (indicated as arrows in Fig. 4.1B) and these PCR products were upregulated in the SOD1 (G93A) transgenic mouse. Sequencing of these PCR products revealed that they contained portions of rRNA sequence from primer sites followed by unknown sequence regions (USR) and poly(A) as schematically demonstrated in Figure 1B. For 18S, the rRNA sequence ends at +1700 followed by 31 bp USR (GenBank accession no. AY248756); for 28S, the rRNA sequence ends at +4268 followed by 470 bp USR (GenBank accession no. AY248755). Both unknown sequences contained polyadenylation signals prior to the poly(A) (Fig. 4.1C, double underline). These results suggested that the upregulated RNAs contain portions of 18S or 28S rRNA sequence.

To examine whether the transcripts corresponding to the identified PCR products contained the RDA fragment regions (clone 18S, 28S-1 or 28S-2), we performed PCRs using the same RT products as templates. A PCR product of the expected 1,318 bp size was obtained when using primers A (corresponding to clone 18S) and F (corresponding to 18S USR), indicating that the corresponding transcript contained the clone 18S region. Likewise, a PCR product of the expected 750 bp size was obtained when using
primers D (corresponding to clone 28S-2) and G (corresponding to 28S USR), but no product was obtained when using primers C (corresponding to clone 28S-1) and G, indicating that the corresponding transcript contained only the clone 28S-2 region. The clone 28S-1 may correspond to a different transcript.

To obtain full-length transcripts, we performed the 5′ RLM-RACE (RNA ligase-mediated rapid amplification of cDNA end). Only authentic capped 5′ ends of transcript are detected by RLM-RACE. We determined that the transcript corresponding to the identified clone 18S is a capped and polyadenylated transcript with virtual 100% homology to the 18S rRNA from +1 to +1700, followed by a 31 bp 3′ terminal end containing a polyadenylation signal (Fig. 4.1C). We named this transcript MSUR1 (mutant SOD1 (G93A)-upregulated RNA 1). We were not able to determine the full-length transcript corresponding to 28S-2 by the presented approach. This is likely due to its larger sizes (4.5-5.5 kb) as determined by Northern blot analysis (see below) (This part of data was done by Mr. Michael P. Stockinger).
Figure 4.1 Three clones identified by RDA correspond to capped and polyadenylated RNAs that contain large portions of rRNA sequence. (A) Positions of the three identified clones corresponding to rRNA and the positions of primers used for reverse transcription (oligo dT-T7) and PCR (A, B, C, D, E and Q1). (A)$_5$ and (A)$_4$ indicate positions of stretches of adenine within the rRNA. (B) RT-PCR analyses of mRNA prepared from SOD1(G93A) or nontransgenic spinal cords using primers B/Q1 and E/Q1. (C) The sequence structures of transcripts corresponding to 18S and 28S-2 clones (GenBank accession nos. AY248756 and AY248755). Double underlines indicate polyadenylation signals. USR: unknown sequence regions.
4.4.2 The identified transcripts containing portions of rRNA are upregulated in the spinal cord of SOD1 (G93A) mouse

To confirm that the transcripts corresponding to the clone 18S, 28S-1 and 28S-2 were truly upregulated in the spinal cord of the SOD1(G93A) mouse, we performed Northern blot analysis. Highly purified poly(A)$^+$ RNA prepared from spinal cords of 2-month-old SOD1(G93A) mice or nontransgenic siblings were analyzed initially using RNA probes prepared from clone 18S, 28S-1 or 28S-2. As shown in Figure 4.2A, the 18S probe hybridized to a major 1.8 kb-band, the 28S-1 probe hybridized to a 5 kb-band and many bands of smaller size, and the 28S-2 probe hybridized to a 5 kb-band and also many bands of smaller size. The 18S 1.8 kb-band, 28S-1 5kb-band and 28S-2 5 kb-bands as well as some of the smaller bands were upregulated in the SOD1 (G93A) spinal cord as compared to the nontransgenic spinal cord. This upregulation was not observed in unaffected tissues, such as the kidney and brain, or in the spinal cords of transgenic mice overexpressing wild-type human SOD1 (data not shown). The equal loading of poly(A)$^+$ RNA samples was confirmed by probing with a β-actin probe (Fig. 4.2A). Furthermore, the β-actin probed blot showed a well-defined single band, indicating that the multiple bands were not degradation products. The specificity of the 18S, 28S-1, and 28S-2 signals was confirmed by their presence even after very high stringency washing as well as by virtue of their being greatly diminished on blots pre-hybridized with unlabeled antisense RNA prepared from the clones (Fig. 4.2A, MB). The observed signals were not the result of incomplete 18S or 28S rRNA removal during the poly(A) selection, since the signal intensities of the probes derived from the different segments of 28S rRNA
were not equal. Additionally, the banding patterns from the two probes were different, thereby suggesting that the observed signals were not the result of rRNA contamination of the poly(A)+ RNA pool. We further probed the blots with RNA probes prepared from the unknown sequence regions (USR). As shown in Figure 4.2B, the 18S-USR probe hybridized to a 1.7-1.8 kb-band and the 28S-2-USR probe hybridized to a 4.5-5.0 kb-band. These bands were upregulated in the SOD1 (G93A) spinal cord, as compared to the nontransgenic spinal cord. Densitometric analysis showed that transcripts corresponding to 18S, 28S-1, and 28S-2 were increased about 2.3-2.0- and 2.1-fold, respectively, in the SOD1 (G93A) spinal cord (Fig. 4.2C). The above results led us to speculate that there may be an overlooked group of capped and polyadenylated transcripts containing large portions of rRNA sequence. We searched the mouse EST database of GenBank using full sequences of both 18S and 28S rRNA (GenBank accession nos. X00686 and X00525, respectively). The initial search found more than 500 clones containing portions of 18S rRNA and more than 1,000 clones which contain portions of 28S rRNA. Further analysis revealed that these types of transcripts are not only widely expressed in tissues from many different organs, but are also expressed at multiple development stages and in diverse organisms. (This part of data was done by Mr. Michael P. Stockinger.)
Figure 4.2 Northern blot analyses indicate that polyadenylated RNAs containing portions of rRNA are upregulated in the spinal cord of SOD1(G93A) mouse. Each lane was equally loaded with 0.5 µg of poly(A)$^+$ RNA prepared from 8 weeks old SOD1(G93A) or nontransgenic spinal cords. The blots were probed with RNA probes that were prepared from clone 18S, 28S-1 or 28S-2 (A). The blots were also probed with RNA probes that were prepared from the unknown sequence regions (B). A total of 6 SOD1 transgenic and 6 nontransgenic sibling mice were examined in three independent experiments with consistent results. (C) Densitometric analysis of Northern blot results. *P<0.001
4.4.3 MSUR1 rescues SOD1(G93A)-mediated cell death in HEK293 cells as well as in NSC34 cells

To explore the possible function of the identified transcript MSUR1, we transiently transfected HEK293 cells with different amounts of pcDNA3/MSUR1 but the same amount of pcDNA3/SOD1 (G93A). We observed that cells transfected with pcDNA3/SOD1 (G93A) alone started to detach from the dish at ~48 hr after transfection. By 72 hr post-transfection, a large number of transfected cells were detached. Trypan blue staining of these floating cells indicated that they were either dead or dying. This phenomenon was not observed in the cells transfected with pcDNA3/wild-type SOD1. Significantly, MSUR1 prevented SOD1 (G93A)-mediated cell death in a dose-dependent manner (Fig. 4.3A&B). Western blot analysis showed that the expression of SOD1 (G93A) protein was the same in all samples (Fig. 4.3B), indicating this protective effect was specifically due to MSUR1 expression.

To further confirm the observed protective effect, we generated HEK293 cell lines stably expressing MSUR1. Seven individual cell lines were established. The levels of MSUR1 mRNA were determined for each individual cell line by quantitative RT-PCR. Human SOD1 mRNA was used as an internal control. Clones C and F, which expressed the lowest and the highest MSUR1 mRNA levels respectively, were selected for further analysis (Fig. 4.3C). We transiently transfected Clone C, Clone F and HEK293 cells with the same amount of pcDNA3/SOD1 (G93A) and then measured cell viability at 72 hr post-transfection. Clone F cells, which expressed more MSUR1, were more resistant to SOD1 (G93A)-mediated cell death (Fig. 4.3D). The cell viability was correlated with the
level of MSUR1 expression. These results were consistent with the co-expression experiments (Fig. 4.3B).

Increases in free radicals have been reported in tissues from transgenic mice expressing the catalytically active mutant SOD1 (G93A), which is considered to be one of the toxicities of mutant SOD1(G93A) (Andrus et al., 1998; Hall et al., 1998). We examined free radical levels for each of the cell lines transfected with pcDNA3/SOD1 (G93A) at 48 hr post-transfection by dichlorofluorescence (DCF) assay. The nonfluorescent fluorescin derivative (dichlorofluorescin, DCFH₂), after being oxidized by free radicals, becomes DCF and emits fluorescence. Clone F cells had a significantly lower free radical level than Clone C or HEK293 cells (Fig. 4.3E). Moreover, we measured protein carbonyl contents for each of the samples to determine the levels of protein oxidation. Clone F cells had significantly less protein oxidation than Clone C or HEK293 cells (Fig. 4.3F). These results indicated that MSUR1 was able to reduce SOD1 (G93A)-mediated free radical levels and oxidative damage.

NSC34 cells are hybrid mouse motor neuron/neuroblastoma cells that retain the ability to proliferate while exhibiting many motor neuron characteristics (Cashman et al., 1992; Durham et al., 1993; Menzies et al., 2002). To examine whether the observed protective effect occurs in NSC34 cells, we generated NSC34-MSUR1 stable cell lines and also NSC34-luciferase stable cell lines as a control. Consistent with the observation in HEK293 cells, NSC34-MSUR1 cells were more resistant to SOD1 (G93A)-mediated cell death when compared to NSC34-luciferase cells (Fig. 4.3G). These results indicated that MSUR1 was able to reduce SOD1 (G93A)-mediated toxicity and the consequent cell death.
Figure 4.3 MSUR1 reduces SOD1 (G93A)-mediated toxicity and cell death in HEK293 cells as well as in NSC34 cells. HEK293 cells were transiently transfected with indicated plasmid DNAs and then harvested for analysis at 72 hr post-transfection. (A) Bright-field microscopy of transfected cells showed that MSUR1 had a protective effect on SOD1 (G93A)-induced cell death. (B) The number of viable cells (assessed with cellular protein concentrations) was increased with increasing amount of MSUR1 cDNA transfected into cells (*P<0.002; **P<0.0001). The ratio of attached cells to dead cells was normalized to the cellular protein concentration of luciferase cDNA-transfected cells. Western blot analysis showed that the expression of SOD1 (G93A) protein was the same in all samples. (C) RT-PCR analysis of MSUR1 level in HEK293 stable lines showed that Clone F expresses more MSUR1 than Clone C. (D) The indicated cell lines were transiently transfected with 1µg of pCDNA/SOD1 (G93A) and then harvested for determining cell viability at 72 hr post-transfection. The cell viability was correlated with the level of MSUR1 expression (*P<0.01; **P<0.001). (E) Hydroxyl radical levels were examined at 48 hr post-transfection with pcDNA3/SOD1 (G93A) by DCF assay. The hydroxyl radical level was reversely correlated with the level of MSUR1 expression (*P<0.005; **P<0.0001). (F) Protein oxidation levels were examined at 48 hr post-transfection with pcDNA3/SOD1 (G93A) by measuring protein carbonyl contents. The protein oxidation level was reversely correlated with the level of MSUR1 expression. Equal loading was confirmed by Ponceau S staining and by probe with anti-β-actin antibodies. (G) NSC34 stable lines were transiently transfected with indicated plasmid DNAs and then harvested for determining cell viability at 72 hr post-transfection. MSUR1 had a protective effect on SOD1 (G93A)-induced cell death in NSC34 cells (*P<0.001).
Figure 4.3
Figure 4.3 continued
4.4.4 MSUR1 reduces hydrogen peroxide-induced cytotoxicity in HEK293 cells as well as in NSC34 cells

We investigated whether MSUR1 has protective activity against the cytotoxicity produced by exposure to hydrogen peroxide (H$_2$O$_2$) in HEK293 cells. H$_2$O$_2$ has been frequently used in cell culture models to induce oxidative stress, as it is capable of altering the intracellular redox state by converting itself to highly reactive hydroxyl radicals (Halliwell et al., 2000; Sokolova et al., 2001). HEK293, Clone C and Clone F cells were treated with H$_2$O$_2$ for 30 min and then examined for free radical levels by DCF assay, as well as for oxidized protein levels by measuring protein carbonyl content. Clone F cells, which expressed more MSUR1, had the least free radical and protein oxidation levels among three cell lines (Fig. 4.4A&B). Furthermore, MTT assay was performed to measure mitochondria activity for these three cell lines after H$_2$O$_2$ treatment. The results showed that after H$_2$O$_2$ treatment, Clone F cells had significantly higher mitochondrial function as compared to Clone C or HEK293 cells (Fig. 4.4C). We also performed H$_2$O$_2$ treatment experiments on NSC34 cells. Similar results were obtained, in which NSC34-MSUR1 cells had a significantly lower free radical level (Fig. 4.4D) and higher mitochondrial function (Fig. 4.4E), as compared to NSC34-luciferase cells. These results indicated that MSUR1 was able to reduce H$_2$O$_2$-induced cytotoxicity.
Figure 4.4 MSUR1 reduces hydrogen peroxide-induced cytotoxicity in HEK293 cells as well as in NSC34 cells. (A) HEK293 stable lines were treated with indicated concentration of H$_2$O$_2$ for 30 min and the hydroxyl radical levels were evaluated by DCF assay. Clone F cells had significant less hydroxyl radical level than Clone C or HEK293 cells. (B) Protein oxidation levels were examined at 3 hr after 700 µM H$_2$O$_2$ treatment by measuring protein carbonyl contents. Clone F cells had significant less protein oxidation compared to Clone C or HEK293 cells. Equal loading was confirmed by Ponceau S staining and by probe with anti-β-actin antibodies. (C) Mitochondrial functions were measured by MTT assay at 3 hr after 700 µM H$_2$O$_2$ treatment. Clone F cells had significant higher mitochondrial function compared to Clone C or HEK293 cells (*$P<0.01$; **$P<0.001$). (D) NSC34 stable lines were treated with indicated concentration of H$_2$O$_2$ for 30 min, and the hydroxyl radical levels were evaluated by DCF assay. NSC34-MSUR1 cells had significant less hydroxyl radical level than NSC34-luciferase cells. (E) Mitochondrial functions were measured by MTT assay at 3 hr after H$_2$O$_2$ treatment. NSC34-MSUR1 cells had significant higher mitochondrial function compared to NSC34-luciferase cells (*$P<0.001$).
Figure 4.4
Figure 4.4 continued

D

DCF Assay (Fluorescence intensity, F30-F0/µg protein)

400 µM 700 µM H₂O₂

NSC34-MSUR1 NSC34-luciferase

E

Mitochondrial Function Assay (M11 assay, arbitrary units)

0 100 400 700 µM H₂O₂

NSC34-luciferase NSC34-MSUR1
4.4.5 MSUR1 does not encode a protein, suggesting a functional non-coding RNA

MSUR1 is an unusual RNA. It has a typical mRNA structure with a cap at the 5′ end and poly (A) at 3′ end, but is, essentially, a portion of 18S rRNA. We investigated whether MSUR1 encodes a protein accounting for the observed protective effect. There are eight potential open reading frames (ORFs 1-8) presented on MSUR1 cDNA sequence (Fig. 4.5A). To determine which ORF could encode MSUR1 protein, we prepared two deletion constructs: deletion #1, which deleted +1 to +591 resulting in missing ORF1-3, and deletion #2, which deleted +1 to +794 resulting in missing ORF1-3 and part of ORF4 (Fig. 4.5A). Each construct was then co-transfected with pcDNA3/SOD1 (G93A) into HEK293 cells, and the protective function was assessed by measuring cell survival and protein oxidation levels. The results showed that deletion #1 still had a protective function equivalent to full length MSUR1, but deletion #2 almost entirely lost protective function (Fig. 4.5B&C). To further confirm the results, we generated NSC34-deletion #1 and NSC34-deletion #2 stable lines. These cell lines were treated with H₂O₂ and then measured for free radical level by DCF assay. NSC34-deletion #1 cells had a significantly lower free radical level compared to NSC34-luciferase cells, whereas NSC34-deletion #2 cells had only a slightly lower free radical level compared to NSC34-luciferase cells (Fig. 4.5D). The difference between deletion #1 construct and deletion #2 construct is that the latter one destroyed ORF4, suggesting that ORF4 might be the one encoding for MSUR1 protein. However, the fact that the deletion #2 construct did not completely lose protective function suggested that ORF5 could not be ruled out.
We performed *in vitro* translation to further assess whether MSUR1 encodes a protein. This was carried out in a coupled transcription/translation rabbit reticulocyte lysate system, using pcDNA3/MSUR1 and pcDNA3/deletion #1 plasmid DNAs. The expected molecular weights were ~3.9-kDa for ORF4 and ~5.9-kDa for ORF5. Thus, the products were analyzed by acrylamide gel electrophoresis under conditions which are able to resolve peptides as small as 2.5-kDa. As shown in Figure 5E, no product was detected in full length MSUR1 or deletion #1 sample, but the positive controls, including SMN protein (32-kDa) and luciferase protein (75-kDa; not shown) were properly produced. These results suggested that MSUR1 might not encode a protein. To further confirm this, we carried out site-directed mutagenesis experiments. A stop codon was introduced at the second and the third amino acids of ORF4 and ORF5, respectively. Each mutant cDNA was then co-transfected with pcDNA3/SOD1 (G93A) into HEK293 cells, and the protective function was assessed by measuring cell survival and protein oxidation level. The results showed that both mutants still had as much protective function as full length MSUR1 (Fig. 4.5F shows the result of protein oxidation). Taking these results together, we concluded that MSUR1 is a functional non-coding RNA.
Figure 4.5 MSUR1 does not encode a protein, suggesting a functional non-coding RNA. (A) Positions of 8 potential open reading frames (ORF) and structures of two deletion constructs. (B&C) HEK293 cells were transiently transfected with indicated plasmid DNAs and then harvested for determining cell viability at 72 hr post-transfection (B) and for measuring protein oxidation level at 48 hr post-transfection (C). Deletion #1 had a protective function as full length MSUR1, but deletion #2 almost entirely lost protective function (*P<0.0002). (D) NSC34 stable lines were treated with indicated concentration of H$_2$O$_2$ for 30 min, and the hydroxyl radical levels were evaluated by DCF assay. NSC34-deletion #1 cells had significant less hydroxyl radical level than NSC34-deletion #2 or NSC34-luciferase cells (*P<0.0001). (E) In vitro translation showed that MSUR1 or deletion #1 did not produce any detectable protein product, whereas the control SMN cDNA produced the expected protein product. (F) A stop codon was introduced at the second and the third amino acids of ORF4 (ORF4M) and ORF5 (ORF5M), respectively. Each mutant cDNA was then co-transfected with pcDNA3/SOD1(G93A) into HEK293 cells and determined protein oxidation levels. Both mutants still had protective function as full length MSUR1. Equal loading was confirmed by Ponceau S staining and by probe with anti-β-actin antibodies.
4.4.6 MSUR1 is widely expressed in various tissues and up-regulated in response to mutant SOD1 (G93A)-mediated toxicity, but not to H2O2-, glutamate- or Aβ-mediated toxicity

MSUR1 expression profile was examined in multiple tissues of 60-day old wild-type mice, including the brain, spinal cord, heart, liver, spleen, lung, kidney, and muscle, by RT-PCR analysis. The results showed that MSUR1 is widely expressed (Fig. 4.6A).

Next, we investigated whether MSUR1 is up-regulated in response to various toxicities mediated by mutant SOD1 (G93A), H2O2, glutamate or Aβ1-42. Primary cortical dissociate cultures were used in this study. The 7-day old cultures were treated with 100 µM H2O2, 20 µM glutamate or 3.3 µM Aβ1-42 for 16 hr and harvested for analysis. For the mutant SOD1 (G93A) study, the 2-day old cultures were transfected with pcDNA3/SOD1 (G93A) or pcDNA3/EGFP and harvested at 72 hr post-transfection. MSUR1 levels were determined by quantitative RT-PCR analysis. As shown in Figure 6B, there was no significant difference in MSUR1 levels among DMEM control, H2O2, glutamate, and Aβ1-42 treated cells. There was ~45% up-regulation of MSUR1 level in pcDNA3/SOD1(G93A) transfected cells compared to pcDNA3/EGFP transfected cells (Fig. 4.6C), which is consistent with the mouse study.
Figure 4.6 MSUR1 is widely expressed in various tissues and up-regulated in response to mutant SOD1 (G93A)-mediated toxicity. (A) RT-PCR analysis of MSUR1 expression in indicated tissues showed that MSUR1 is widely expressed. (B&C) Primary cortical dissociated cultures were treated with 100 µM H₂O₂, 20 µM glutamate or 3.3 µM Aβ1-42 for 16 hr or transfected with pcDNA3/SOD1 (G93A) or pcDNA3/EGFP and harvested at 72 hr post-transfection. MSUR1 levels were determined by quantitative RT-PCR analysis. There was no significant difference in MSUR1 levels among DMEM control, H₂O₂, glutamate, and Aβ1-42 treated cells. There was ~45% up-regulation of MSUR1 level in pcDNA3/SOD1 (G93A) transfected cells compared to pcDNA3/EGFP transfected cells (*P<0.001). Aβ1-42 was pre-incubated at 37°C for 24 hr prior to adding it to the cultures.
4.5 Discussion

In the present study, we utilized representational difference analysis to identify the transcripts which are upregulated or newly induced in the pre-symptomatic stage of SOD1 (G93A) mice. Unexpectedly, three of our identified clones were rRNA segments, and they were the predominant clones found at 4 and 8 weeks of age. Upon further analysis of one of these clones, it was discovered that it corresponds to capped and polyadenylated transcripts containing long segments of sequence with 100% homology to 18S rRNA (MSUR1; Fig. 4.1). There are hundreds of known mRNAs containing short (20-50 nucleotides) rRNA sequences, an example of which is mouse IgE-binding factor mRNA, which contains 13 nucleotides of 18S (GenBank accession no. M10062). Mauro et al. have shown that rRNA-like sequences occur in a wide variety of primary transcripts. They hypothesized that these rRNA-like sequences may function as cis-regulatory elements that regulate translational efficiency by interacting with rRNA or ribosomal proteins. Only a few known mRNAs contain long rRNA sequences. For example, mouse heat shock 86 protein mRNA contains 229 nucleotides of 28S (GenBank accession no. J04633) (Mauro and Edelman, 1997). However, mouse EST database analysis indicates that there is a high abundance of uncharacterized polyadenylated RNAs that contain several hundred bases of rRNA sequence. The reason why only a few these types of transcripts have been identified may be because they are easily disregarded as experimental artifacts resulting from rRNA contamination of the poly (A)$^+$ RNA pool.

What are the origins of these types of transcripts? Some of these RNAs may derive from genes which contain long rRNA sequences. It is possible that some of them may derive from rRNA. Some rRNAs may process to become mature mRNAs,
which may have specific functions or may encode functional proteins. For example, the Humanin mRNA is 100% homologous to 16S rRNA gene and encodes a short polypeptide (Hashimoto et al., 2001). Humanin has been shown to rescue neuronal cell death caused by multiple different types of familial Alzheimer’s disease genes (Hashimoto et al., 2001). The rat homologue of Humanin, named Rattin, also displays protective activity against excitotoxic neuronal death (Caricasole et al., 2002; Maximov et al., 2002). Maximov et al. have suggested that Humanin mRNA may be transcribed directly from the 16S gene but translated in the cytosol (Caricasole et al., 2002; Maximov et al., 2002). The origins of these types of transcripts remain to be elucidated. It is possible that some transcripts of this type may have functional significance for some neurodegenerative disease processes, which will be investigated in the future.

One important finding from this study is that the identified MSUR1 has protective effects on SOD1 (G93A)-mediated cell death in both HEK293 cells and NSC34 cells (Fig. 4.3), suggesting that MSUR1 may be a rescue factor which is upregulated against SOD1(G93A)-induced cell damage during the pre-symptomatic stage of the disease process in mice. In vivo, mutant SOD1 expression in multiple cell types, including microglia, astrocytes, oligodendrocytes and motor neurons, is necessary to cause motor neuron death. The upregulation of MSUR1 in mice might be a response of one or more cell types. It is possible that MSUR1 might play an important role in preventing motor neuron degeneration at an early age of mice. It will be worthwhile to investigate if over-expression of MSUR1 would delay the disease onset and life span of SOD1 (G93A) mice. In addition, whether MSUR1 is also up-regulated in other mutant SOD1 mice and
whether MSUR1 has protective effects on other SOD1 mutants remains to be investigated.

We observed that SOD1 (G93A)-induced free radicals were significantly reduced in the cells expressing MSUR1 (Fig. 3). Further, MSUR1 could also reduce $\text{H}_2\text{O}_2$-induced cytotoxicity in both HEK293 cells and NSC34 cells (Fig. 4.4). It appears that the function of MSUR1 might be involved in oxidative stress. The mechanisms underlying the observed protective effects by MSUR1 remain to be elucidated.

*In vitro* translation and site-directed mutagenesis analysis suggests that MSUR1 is a functional non-coding RNA (Fig. 4.5). More and more non-coding RNAs (ncRNAs) have been found to have essential functions in many pathophysiology processes other than simple intermediates in between DNA transcription and protein synthesis. According to their size, they can be classified into microRNA (~20 bp), small RNA (~20-300 bp), and medium or large sized RNA (over ~300bp, up to or over 10,000). Functions of medium or large sized RNA includes gene silencing, gene transcription, DNA imprinting, RNA interference, tumor suppressor, and relation to stress or apoptosis (Costa, 2005; Mattick and Makunin, 2006; Costa, 2007). For example, heat shock RNA-1 (HSR1) is a co-transcriptional factor activator, along with elongation factor eEF1A to activate heat-shock transcription factor 1 (Shamovsky et al., 2006). Furthermore, several non-coding RNAs involved in different central nervous system diseases have been identified in the last decades. For example, Prion diseases are primarily caused by an infectious protein designated PrP(Sc). The conversion of the normal PrP(C) to PrP(Sc) is the central pathogenic event. Two studies demonstrated that Prion associated RNAs are required to stimulate efficient PrP(SC) production and cause the pathological aspects of the diseases (Deleault et al., 2003; Supattapone, 2004). In our case, our hypothesis for the
possible protective function is that MUSR1 might bind to some specific transcriptional factors, or may act by a RNA-DNA interaction, thereby upregulating some antioxidant gene expression, enhancing the cellular antioxidant capacity. It is also possible that MSUR1 might directly interact with mutant SOD1 to affect mutant SOD1 activity and toxicity. Another possibility is that MSUR1 might protect mitochondria from generating excess free radicals or preventing mutant SOD1 entering the intermembrane space. The mechanisms underlying the protective functions will be investigated in the future.

RT-PCR analysis showed that MSUR1 is widely expressed in various tissues (Fig. 4.6A), indicating its protective function is not limited to cells in the central nervous system. Further, MSUR1 is upregulated in primary cortical dissociated cultures transfected with SOD1 (G93A) cDNAs (Fig. 4.6B), which supports the observations in mice. However, we did not observe obvious induction in the cultures treated with H$_2$O$_2$, glutamate or A$\beta$1-42, suggesting that the induction may be specific to SOD1 (G93A)-mediated toxicity.

In summary, we presented a class of polyadenylated RNA that contain large portions of 18S or 28S rRNA sequence. MSUR1 belongs to this family of transcripts. Importantly, we demonstrated that MSUR1 has protective functions against mutant SOD1 (G93A) toxicity, implicating that MSUR1 may be a potential therapeutic target for ALS.
CHAPTER 5

CONCLUSION AND PERSPECTIVES

Amyotrophic Lateral Sclerosis (ALS) is a late-onset, devastating fatal neurodegenerative disorder that is characterized by progressive degeneration of motor neurons in the spinal cord, motor cortex and brainstem, resulting in muscle weakness, atrophy, and spasticity. The pathogenesis of ALS largely remains unknown. Oxidative stress is considered to be an important contributor to the disease. There is a large body of evidence implicating oxidative damage in the pathogenesis of ALS. Recent studies have demonstrated that RNA oxidation is significantly increased in vulnerable neurons of Alzheimer’s disease (AD) as well as in substantia nigra neurons of Parkinson disease (PD). The first part of this dissertation investigates the following issues in ALS postmortem tissues and in ALS transgenic mice SOD1 (G93A): (1) whether increased RNA oxidation occurred in ALS pathogenesis, (2) what kind of RNA has been oxidized, (3) whether mRNA is selectively or randomly oxidized, (4) if oxidation is selective, what kind of mRNA species are oxidized, (5) at which stage of the disease and in what kind of cells have increased RNA oxidation, (6) whether RNA oxidation contributes to the disease. In addition to SOD1 (G93A) transgenic mice, RNA oxidation levels were also examined in several other mutant SOD1 transgenic mice, including G37R, G85R, G127X,
and His46R/His48Q.

This dissertation demonstrates that in SOD1 (G93A) transgenic mice, there is significantly increased RNA oxidation in motor neurons and oligodendrocytes of the spinal cord at an early pre-symptomatic stage (2-month-old) when the motor neurons still appear healthy. Identification of oxidized mRNA species revealed that certain mRNA species are more susceptible to oxidative damage; thus, RNA oxidation is not random but highly selective. Identification of oxidized RNA species revealed that some mRNA species, such as mRNAs encoding for mitochondrial proteins or translational machinery proteins, were more vulnerable to oxidative damage. The expression levels of proteins corresponding to the oxidized mRNA species were significantly decreased. Vitamin E reduced RNA oxidation in motor neurons. Vitamin E delays disease onset about two weeks, improves motor performance, but does not alter survival. Vitamin E protects the motor neurons from cell death, reduces the extent of ubiquitin aggregation, gliosis, and mitochondrial vacuolization. RNA oxidation, itself, is directly associated with neuronal deterioration and is not simply a coincidental epiphenomenon during the process of neurodegeneration. Moreover, the increased oxidized mRNA was also detected at pre-symptomatic stage in other ALS human mutant SOD1 transgenic mice expressing inactive human SOD1 activities, such as H46R/H48Q, G85R, G127X, suggesting that increased RNA was not merely due to human SOD1 overexpression.

Studies of human ALS postmortem tissue revealed that varied amounts of mRNA have been oxidized in the motor cortex and lumbar spinal cord of both sporadic and familial ALS patients, but not in normal patients or in ALS disease-spared tissues. Further identification of the oxidized mRNA species, using microarray analysis,
showed that mRNA oxidation is not random but is highly selective, even at the end stage of the disease. Compared to SOD1 (G93A) transgenic mouse array, the human ALS array includes more categories of oxidized mRNA corresponding proteins’ activity ranging from apoptosis, cell cycle control, immune response, and transcription, etc, reflecting an end stage decompensate state of the disease.

In this dissertation, using microarray analysis, the profile of oxidized mRNAs species in 60-day SOD1 (G93A) transgenic mice was characterized as well as the profile of oxidized mRNA species in the human ALS motor cortex and spinal cord. A very striking finding of microarray is that many of the identified oxidized transcripts are those of genes known to be related to ALS. These oxidized mRNAs’ corresponding proteins may result in compromised normal functions, reduced expression level, or potentially the gain of some toxic functions.

Surprisingly, comparison oxidized mRNA species profiles between ALS motor cortex array and AD frontal cortex array revealed that the oxidized mRNA species of these two neurodegenerative diseases are quite similar; suggesting that these oxidized mRNAs at the end stage of diseases might play a role in accelerating the process of neurodegeneration.

Taken together, these studies suggest that mRNA oxidation may be a critical factor initiating the cascade of motor neurons degeneration. Antioxidant therapeutic intervention at the earliest possible stage of the disease may be beneficial.

The second part of this dissertation is an investigation of a novel non-coding RNA which can rescue mutant SOD1-mediated cell death. MSUR1 belongs to a class of polyadenylated RNA that contain large portions of 18S or 28S rRNA sequence.
Importantly, MSUR1 has protective functions against mutant SOD1 (G93A) toxicity in cell culture, implicating that MSUR1 may be a potential therapeutic target for ALS. It will be worthwhile to investigate if over-expression of MSUR1 *in vivo* would delay the disease onset and prolong the life span of SOD1 (G93A) mice. And further elucidation of the mechanism of this rescue may help us to better understand these kinds of transcripts.
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