HoxB2 binds mutant SOD1 and is altered in transgenic model of ALS

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Mutations in Cu/Zn superoxide dismutase (SOD1) cause ~20% of familial amyotrophic lateral sclerosis by a toxic gain of function; however, the precise mechanisms remain unclear. Here, we report the identification of HoxB2, a homeodomain-containing transcription factor, as a G93A mutant SOD1 interactive protein in a yeast two-hybrid screen. We show that HoxB2 co-precipitates and co-localizes with mutant SOD1 in neuronal cell lines, as well as in brain and spinal cord of G93A mutant SOD1 transgenic mice. Mutagenesis further shows that this interaction is mediated by the central homeodomain of HoxB2. In motor neuron-like NSC-34 cells, overexpression of HoxB2 or its homeodomain decreases the insolubility of mutant SOD1 and inhibits G93A or G86R mutant SOD1-induced neuronal cell death. In human and mouse tissues, we show that expression of HoxB2 persists in adult spinal cord and is primarily localized in nuclei of motor neurons. In G93A transgenic mice, HoxB2 co-localizes with mutant SOD1 and is redistributed to perikarya and proximal neurites of motor neurons. In addition, there is progressive accumulation of HoxB2 and mutant SOD1 as punctate inclusions in the neuropil surrounding motor neurons. Taken together, our findings demonstrate that interaction of HoxB2 with mutant SOD1 occurs in motor neurons of G93A mutant SOD1 transgenic mice and suggest that this interaction may modulate the neurotoxicity of mutant SOD1.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) occurs in familial (fALS) and sporadic (sALS) forms and is characterized by a late onset, progressive loss of motor neurons, leading to paralysis and death. Approximately 20% of fALS are associated with missense mutations in a widely expressed Cu/Zn superoxide dismutase (SOD1) (1). The missense mutations are believed to destabilize the protein and lead to misfolded and neurotoxic aggregation of the mutant SOD1 protein. However, how mutations in SOD1 proteins cause motor neuron degeneration is poorly understood. Recent studies have implicated transcriptional alterations in the pathogenesis of ALS (2,3).

Hox genes are a family of master regulatory genes controlling embryonic morphogenesis. They encode transcriptional factors whose expression during development alters the fate of pluripotential cells and provide the genetic template for axial segmentation and positional specification, thereby establishing the embryonic anlage of the adult body plan (4). In higher vertebrates, Hox genes have evolved into four clusters (HoxA, HoxB, HoxC and HoxD) by tandem duplication from a single ancestral cluster (5). Each cluster contains similar sets of duplicated or paralogous genes whose spatial and temporal collinear expressions during development confer complementary and overlapping functions.

Less attention has been directed at the role of Hox genes in the subsequent development of body parts and organs. However, similar spatial and temporal collinear expressions of Hox genes underlie the symmetrical formation of fore- and hindlimbs (6), as well as the development of hematopoietic tissues (7,8), skeletal muscle (9), skin (10), intestine (12,13), pancreatic islet beta- and alpha-cells (14), oocyte (15) and female and male reproductive tracks (16,17). Hox genes are particularly instrumental in the developing nervous system. Their expression establishes the rostrocaudal axis in embryonic hindbrain and spinal cord (18) and is a major...
determinant of motor neuron identity in brainstem and ventral spinal cord (19).

Many Hox genes are also expressed in fully differentiated adult tissues and cells (20,21) and undergo dysregulation in neoplastic cells (22). These observations have led to the hypothesis that Hox genes provide genetic input for maintaining as well as establishing differentiated states. In support of this view, we herein report HoxB2 as an interactive partner of mutant SOD1. We show that HoxB2 continues to be expressed in mature brain and spinal cord and associates with mutant SOD1 in neuronal cell lines and in motor neurons of a transgenic model of mutant SOD1-linked fALS. In motor neuron-like NSC-34 cells, overexpression of HoxB2 alters the insolubility of mutant SOD1 and rescues cells from mutant SOD1-induced neuronal cell death. In transgenic mice, HoxB2 co-aggregates with G93A mutant SOD1 during development of the pathological alterations in motor neurons. The findings suggest that interactions with HoxB2 may modulate the neurotoxic properties of mutant SOD1 on motor neurons.

RESULTS

Identification of HoxB2 as a binding partner for mutant SOD1

To explore the abnormal interactive properties of mutant SOD1 proteins, G93A mutant SOD1 was first fused to GAL4 DNA-binding domain (GAL4 BD) and used as bait in a yeast two-hybrid screen of a mouse embryonic spinal cord cDNA library. More than 6.5 × 10^7 colonies were screened and no positive clone was identified. As native SOD1 is a homodimer, fusion of GAL4 BD might have impaired the dimerization of G93A mutant SOD1 in yeast and interfered with the interactions of G93A mutant SOD1 with other proteins. Therefore, G93A mutant SOD1 homodimers were constructed by linking two G93A mutant SOD1 monomers with an immunoglobulin A hinge region, as previously described (7). A G93A mutant SOD1 homodimer was then fused to GAL4 BD and used as bait to screen a mouse embryonic spinal cord cDNA library. More than 1.5 × 10^6 colonies were screened and 29 positive clones were identified.

Table 1. Differential interaction of HoxB2 with mutant SOD1 in yeast

| Yeast transformants | Growth in selective media | (-Trp-Leu-His) | (+3-Amino-1,2,4-triazole) |
|---------------------|---------------------------|----------------|--------------------------|
| BD/G93A SOD1 homodimer + AD BD + AD/HoxB2 BD/G93A SOD1 homodimer + AD/HoxB2 BD/wt SOD1 homodimer + AD/HoxB2 | Growth | No growth | Growth |
| No growth | No growth | Growth | No growth |
| BD + AD/HoxB2 BD/G93A SOD1 homodimer + AD/HoxB2 BD/wt SOD1 homodimer + AD/HoxB2 | BD/wt SOD1 homodimer + AD/HoxB2 | BD/G93A SOD1 homodimer + AD BD + AD/HoxB2 | BD + AD/HoxB2 BD/G93A SOD1 homodimer + AD BD + AD/HoxB2 |
| Table 1. Differential interaction of HoxB2 with mutant SOD1 in yeast |

G93A mutant SOD1 and HoxB2 is stronger than that between wild-type SOD1 and HoxB2.

Interactions of SOD1 with HoxB2 in neuronal cell line

To determine whether interactions between SOD1 and HoxB2 occur in neuronal cells, Neuro 2a cells were co-transfected with His6-tagged HoxB2 and unfused GFP, GFP-tagged wild-type SOD1 (homodimer and monomer), GFP-tagged wild-type/G93A heterodimer or GFP-tagged G93A mutant SOD1 (homodimer and monomer). Western blot analyses of cell lysates (50 μg of protein content) showed comparable amounts of HoxB2 and GFP-tagged SOD1 (Fig. 1A). Then, cell lysates (1 mg of protein content) were incubated with Ni-NTA agarose beads. When precipitates were immunoblotted with anti-GFP to assess for interactions with His6-tagged HoxB2, maximal association was observed between HoxB2 and G93A mutant SOD1 (monomer and homodimer) (Fig. 1B, lanes 3 and 6) and between HoxB2 and wild-type/G93A SOD1 heterodimer (Fig. 1B, lane 5). A faint band was seen in the precipitate from Neuro 2a cells co-expressing His6-tagged HoxB2 and GFP-tagged wild-type SOD1 (monomer) (Fig. 1B, lanes 2), indicating that HoxB2 binds weakly to wild-type SOD1. Together, these results demonstrate that HoxB2 associates with mutant SOD1 monomer and homodimer, with SOD1 wild-type/mutant heterodimer and, to a much lesser extent, with wild-type SOD1 monomer and wild-type/wild-type SOD1 homodimer.

Interactions of HoxB2 with different FALS-associated mutant SOD1 proteins were also examined in Neuro 2a cells. As shown by anti-GFP immunoblots of Ni-NTA precipitates, HoxB2 interacts very strongly with monomeric G93A, G86R, G37R, C6F, D90A and L126S mutant SOD1 (Fig. 1C). These results indicate that several FALS-associated mutant SOD1 proteins have enhanced interactions with HoxB2 in Neuro 2a cells.

Co-localization of HoxB2 with mutant SOD1 aggregates in neuronal cell lines

We next assessed the subcellular distributions of HoxB2 and SOD1 in Neuro 2a and NSC-34 cells co-transfected with HoxB2 and GFP-tagged SOD1 (wild-type, G93A or G86R). Forty-eight hours after co-transfection, GFP-tagged wild-type SOD1 was distributed in the cytoplasm of co-transfected cells (Fig. 2A and J) and HoxB2 was distributed in the nuclei and,
to a lesser extent, in the cytoplasm (Fig. 2B and K). In cells co-transfected with GFP-tagged mutant SOD1 proteins (Fig. 2D, G, M and P), HoxB2 (Fig. 2E, H, N and Q) co-localized with mutant SOD1 aggregates in the cytoplasm and nucleus (Fig. 2F, I, O and R). The findings indicate that co-expression of mutant, but not wild-type, SOD1 protein leads to co-localization of HoxB2 with mutant SOD1 aggregates in neuronal cells.

HoxB2 interacts with mutant SOD1 via its homeodomain

We next examined the domains of HoxB2 mediating the binding of SOD1. The common amino acid sequence among the eight cDNAs identified from yeast two-hybrid screen is from amino acids 119–244. This region encompasses the central homeobox, 24 amino acids of N-terminal flanking sequence and 46 amino acids of C-terminal flanking sequence (Fig. 3A). To determine the interactive domain, we generated constructs of HoxB2 interactive fragments with deletions of the N- and/or C-flanking sequences. Lysates from Neuro 2a cells co-transfected with GFP-tagged G93A mutant SOD1 and His6-tagged N- or C-terminal deletions of the interactive fragment were incubated with Ni-NTA agarose beads, and the precipitates were subjected to western blot analysis. As shown in Figure 3B, deletion of 24 amino acids of N-terminal flanking sequence or 46 amino acids of C-terminal flanking sequence did not affect binding of G93A mutant SOD1 to the homeobox domain. However, deletion of both N- and C-terminal flanking sequences reduced binding of homeobox domain to G93A mutant SOD1. These studies support the conclusion that G93A mutant SOD1 binds to the homeodomain of HoxB2, whereas the flanking sequences may enhance the association with mutant SOD1.

HoxB2 decreases the insolubility of mutant SOD1 in NSC-34 cells

Mutant SOD1 aggregates in the spinal cords from mutant SOD1 transgenic mice and certain SOD1-linked fALS patients (24–26), and aggregation of mutant SOD1 associates with loss of detergent solubility (27). We thus used transiently transfected NSC-34 cells as a model to assess the effects of interaction of HoxB2 on solubility of mutant SOD1 protein. Forty-eight hours after transfection, detergent-soluble supernatant and detergent-insoluble pellet fractions were prepared from NSC-34 cells expressing wild-type SOD1, G93A or G86R mutant SOD1, as well as from cells co-expressing G93A or G86R mutant SOD1 and HoxB2, HoxB2 homeodomain or GFP control. Supernatants and pellets were subjected to SDS–PAGE gel electrophoresis and western blot analysis. Similar amounts of cells and protein were present in each preparation. Mutant SOD1 was detected in both supernatant and detergent-insoluble pellet fractions (Fig. 4A and B). When NSC-34 cells were co-transfected with mutant SOD1 and HoxB2, the amounts of mutant SOD1 in the pellet fraction were diminished (Fig. 4A and B, compare lanes 2 and 4). Similarly, co-expression of HoxB2 homeodomain also decreased the detected amount of mutant SOD1 in the pellet fraction (Fig. 4A and B, compare lanes 2 and 6), whereas...
The co-expression of GFP had no effects on the solubility of
mutant SOD1 (Fig. 4A and B, compare lanes 2 and 8).
These results indicate that mutations decrease the solubility
of mutant SOD1 and overexpression of HoxB2 or HoxB2
domains reduces the insolubility of mutant SOD1 in
motor neuron-like NSC-34 cells.

HoxB2 inhibits neurotoxicity of mutant SOD1
in NSC-34 cells
As mutant SOD1 aggregation has been implicated in the patho-
genesis of mutant SOD1-linked FALS (25,28,29), we next
examined the effects of HoxB2 on mutant SOD1-induced
neurotoxicity. NSC-34 cells were transiently transfected with
HoxB2, wild-type or G93A mutant SOD1 or co-transfected
with G93A mutant SOD1 together with HoxB2, HoxB2 home-
domain or a control GFP protein. Empty expression vector for
HoxB2 and GFP was added to equalize amounts of transfected
DNA. Forty-eight hours after transfection, cells were analyzed
by Trypan blue exclusion assay. As shown in Figure 5A, trans-
fection of HoxB2 alone has no effect on NSC-34 cell viability.
Expression of wild-type SOD1 slightly decreases the number of
cells permeable to Trypan blue, in agreement with the
observations that wild-type SOD1 has anti-apoptotic activity
(30). Transfection of G93A mutant SOD1 or co-transfection
of mutant SOD1 with an irrelevant GFP protein leads to high percentages of
dead cells permeable to Trypan blue. Co-transfection of HoxB2 with mutant G93A SOD1 reduces
the level of cell death from ~50% of cultured cells to near
the background levels of 20–25% in untransfected cells. A
similar reduction of cell death is achieved by co-transfecting
mutant SOD1 with HoxB2 homeodomain. Co-expression of
HoxB2 or HoxB2 homeodomain also suppresses G86R
mutant SOD1-induced neuronal cell death (Fig. 5B). Immuno-
blots of cell lysates show similar levels of mutant SOD1,
HoxB2, HoxB2 homeodomain and GFP in transfected cells
(data not shown). Together, these data demonstrate that
expression of HoxB2 and its homeodomain reduce mutant
SOD1-induced neurotoxicity and indicate that the interactions
of HoxB2 with mutant SOD1 can modulate the neurotoxic
effects of mutant SOD1.

We also examined whether HoxB2 protects neuronal cells
against unrelated pro-apoptotic insults due to the addition of
staurosporine. NSC-34 cells were first transiently transfected
with HoxB2. Twenty-four hours after transfection, cells were
incubated with 100 or 500 nM of staurosporine, and after
6 h, cell viability was analyzed by Trypan blue exclusion
assay. Staurosporine treatment of HoxB2-transfected NSC-
34 cells induces 32% of cell death at 100 nM and 41% of
cell death at 500 nM (Fig. 5C). Staurosporine treatment of
mock-transfected cells induces 35% of cell death at
100 nM and 43% of cell death at 500 nM (Fig. 5C). No sig-
nificant difference was found between mock and HoxB2-
transfected cells, indicating that expression of HoxB2 may
not protect NSC-34 cells against staurosporine-induced
apoptosis.
**HoxB2 is expressed in mature brain and spinal cord and interacts with mutant SOD1 in G93A transgenic mice**

As mutant SOD1 causes an adult-onset motor neuron disease, the potential role of HoxB2 in pathogenesis would depend on persistence of HoxB2 expression in adult tissues beyond its well-recognized functions during the development (31), as well as its association with mutant SOD1 in vivo. To assess HoxB2 expression, we conducted RT–PCR analysis on RNA extracted from frozen tissues and showed that HoxB2 is expressed in human adult spinal cord (Fig. 6A, left panel). RNA was also extracted from brain of embryonic day 8.5 (E8.5) mice, from brain stem of embryonic day 15 (E15), postnatal day 1 (P1) and adult mice and from spinal cord of adult mice. RT–PCR analysis showed that high levels of mouse HoxB2 transcript were present in embryonic tissues and P1 brain stem, but lower levels of HoxB2 were also expressed in adult brain stem and spinal cord (Fig. 6A, right panel).

We next examined whether the expression of HoxB2 is altered by mutant SOD1 in neuronal cells. Motor neuron-like NSC-34 cells were first transfected with wild-type, G93A or G86R mutant SOD1 transgenes, and RNA was extracted from transfected cells after 48 h. RT–PCR analysis showed that HoxB2 was expressed in NSC-34 cells and that expression level of HoxB2 was not altered by wild-type or G93A and G86R mutant SOD1 (Fig. 6B, left panel). RT–PCR analysis also showed that in NSC-34 cells transfected with G93A mutant SOD1 for 24, 48 and 72 h, the level of HoxB2 expression did not alter as compared to that at 0 h (Fig. 6B, right panel).

Examination of HoxB2 protein in western blots of mouse brain and spinal cord reveals immunoreactive bands that co-migrate with HoxB2 protein from E13 mouse hindbrain (Fig. 6C). Western blot analysis shows HoxB2 expression in brain and spinal cord of presymptomatic G93A SOD1 transgenic mice, age-matched wild-type SOD1 transgenic mice and non-transgenic mice (Fig. 6C). Taken together, the results demonstrate that HoxB2 continues to be expressed in mature brain and spinal cord tissues.

We then examined whether interactions between mutant SOD1 and HoxB2 occur in brain and spinal cord tissues of presymptomatic G93A transgenic mice. Brain and spinal cord extracts were prepared from presymptomatic G93A transgenic mice and preclarified by incubation with protein A/G plus agarose beads. Precleared brain and spinal cord extracts were then incubated overnight with anti-human SOD1 antibodies, and anti-human SOD1 immunoprecipitates were blotted with anti-HoxB2 antibodies. As shown in Figure 6D, HoxB2 is detected in anti-human SOD1 immunoprecipitates of G93A transgenic mice brain and spinal cord homogenates, indicating that HoxB2 associates with G93A mutant SOD1 in brain and spinal cord of presymptomatic G93A transgenic mice.

**Pathological changes of motor neurons in G93A mutant SOD1 transgenic mice are associated with altered distribution of HoxB2 and co-localization of HoxB2 in mutant SOD1 aggregates**

Anti-HoxB2 immunoreactivity was used to assess alterations of HoxB2 associated with pathological changes in motor neurons in transgenic mice expressing a human G93A mutant SOD1 transgene. In non-transgenic mice (Fig. 7A–C), HoxB2 is markedly enriched in nuclei of large neurons, including nuclei of motor neurons in lumbar spinal cord (Fig. 7A, arrows), sensory neurons in dorsal root ganglia (Fig. 7B, arrow) and Purkinje neurons in the cerebellum (Fig. 7C, arrow). Similar nucleic distribution of HoxB2 in motor neurons in spinal cord (Fig. 7D, arrows), sensory neurons in dorsal root ganglia (Fig. 7E, arrow) and Purkinje neurons in the cerebellum (Fig. 7F, arrow) was found in wild-type SOD1 transgenic mice. In presymptomatic (80 days) G93A mutant SOD1 transgenic mice (Fig. 7G–I), HoxB2 is redistributed from nuclei to perikarya in motor neurons (Fig. 7G, arrow), but not in sensory (Fig. 7H, arrow) or Purkinje (Fig. 7I, arrow) neurons. In motor neurons of presymptomatic G93A mutant transgenic mice, HoxB2 is diffusely distributed in perikarya and extends to proximal neurites (Fig. 7G). In addition, punctate aggregates of HoxB2 can be seen in the neuropil surrounding motor neurons of the ventral horn (Fig. 7G, arrowheads). In some sections of lumbar spinal cord, there are increases in the intensity and number of punctate inclusions containing HoxB2 in the neuropil surrounding motor neurons (Fig. 7I). In symptomatic (120 days) G93A mutant SOD1 transgenic mice, numerous punctate inclusions (Fig. 7K, solid arrowheads) and linear

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**Figure 3.** HoxB2 interacts with mutant SOD1 via its central homeodomain. (A) Schematic diagram of full-length HoxB2 (amino acids 1–356), interactive sequence identified by yeast two-hybrid screen (amino acids 119–244) and mutant interactive sequence lacking C-terminal flanking sequence (amino acids 119–200, termed N-homeobox), N-terminal flanking sequence (amino acids 144–244, termed Homeobox-C) or both C- and N-terminal flanking sequences (amino acids 144–200, termed Homeobox). (B) Anti-GFP immunoblots of Ni-NTA precipitates of Neuro 2a cells co-transfected with GFP-tagged G93A mutant SOD1 and His6-tagged full-length (FL HoxB2), N-homeobox, Homebox-C or Homebox sequence (upper panel). Combined anti-HA and anti-HoxB2 immunoblots of whole cell lysates of Neuro 2a cells transfected with FL HoxB2, N-homeobox, Homebox-C and Homebox (lower panel).

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processes (Fig. 7K, open arrowheads) containing HoxB2 immunoreactivity (Fig. 7K) are present in vacuolated neuropil of lumbar ventral horn. In end-stage (150 days) G93A mutant SOD1 transgenic mice, the HoxB2-positive punctate inclusion (Fig. 7L, arrowheads) and linear processes (Fig. 7L, open arrowheads) are readily apparent due to enhanced vacuolization of neuropil and loss of motor neurons (Fig. 7L).

Confocal microscopy of motor neurons in the lumbar spinal cord of symptomatic G93A mutant SOD1 transgenic mice, the HoxB2-positive punctate inclusion (Fig. 7L, arrowheads) and linear processes (Fig. 7L, open arrowheads) are readily apparent due to enhanced vacuolization of neuropil and loss of motor neurons (Fig. 7L).

Confocal microscopy of motor neurons in the lumbar spinal cord of symptomatic G93A mutant SOD1 transgenic mice revealed co-localization of mutant SOD1 (Fig. 7M) with HoxB2 (Fig. 7N) in the merged image (Fig. 7O) in the perikarya of a motor neuron, in a prominent neurite extension and in numerous punctate inclusions (Fig. 7M–O, arrowheads) in the surrounding vacuolated neuropil. Some punctate inclusions in the immediate vicinity of motor neuron were markedly enlarged and resembled dystrophic neurites (Fig. 7M–O, arrows).

**DISCUSSION**

In the present study, we identify HoxB2 as a G93A mutant SOD1 interactive protein in a yeast two-hybrid screen and demonstrate that this interaction is mediated via the central homeodomain of HoxB2. We also show that expression of HoxB2 persists in motor neurons of adult spinal cord, that HoxB2 co-immunoprecipitates with mutant SOD1 in the spinal cord homogenate from presymptomatic G93A mutant SOD1 transgenic mice and that the disposition of HoxB2 in motor neurons is altered by expression of mutant SOD1 in transgenic mice. In presymptomatic mice, HoxB2 is redistributed from a nuclear to cytoplasmic localization and begins to aggregate with mutant SOD1 as punctate inclusions in the neuropil surrounding motor neurons. Similar punctate inclusions contain mutant SOD1 and have been identified as the earliest ultrastructural alterations of motor neurons in G93A transgenic mice (32). In a separate study, we show that similar inclusions in the neuropil surrounding motor neurons also contain NF-L and p190RhoGEF (Lin et al., manuscript in preparation). The early and progressive involvement of HoxB2 in pathological alterations of G93A transgenic mice suggests that interactions of HoxB2 may modulate the neurotoxic effects of mutant SOD1 on motor neurons. This view is supported by the ability of HoxB2 to alter the solubility and neurotoxicity of mutant SOD1 in motor neuron-like NSC-34 cells.

Interactions of HoxB2 that increase solubility and diminish neurotoxicity of mutant SOD1 protein in NSC34 cells are chaperone-like properties, which may alter the interactive properties of mutant SOD1 in different cellular environments. The neurotoxic effects of protein aggregates are believed to arise during early stages of protein aggregation (33) due to formation of soluble oligomers or protofibrils containing interactive surfaces with potentially toxic properties (34, 35). We have found that RNA-triggered aggregation of NF-L promotes the aggregation and loss of solubility of mutant SOD1 in transfected neuronal cells (36) and that RNA-triggered aggregation of NF-L is associated with co-aggregation of p190RhoGEF in transfected cells and in punctate inclusions in the neuropil surrounding degenerating motor neurons (Lin et al., manuscript in preparation) in mice expressing untranslated NF-L RNA in the 3'UTR of a GFP reporter transgene (37). The presence of HoxB2 along with NF-L, p190RhoGEF and mutant SOD1...
in punctate inclusions of presymptomatic G93A transgenic mice suggests that HoxB2 may alter the interactive properties of mutant SOD1 or the co-aggregative properties of NF-L and p190RhoGEF with mutant SOD1. The aggregates may have toxic properties on motor neurons or, alternatively, may have reactive changes with protective effects.

The prospective role of neurotoxic protein aggregation in the pathogenesis of mutant SOD1-induced motor neuron degeneration has led to a search for interactive targets of mutant SOD1 proteins. Indeed, multiple interactive proteins have been identified, including lysyl-tRNA synthetase and translocon-associated protein delta (23), Dorfin (38),

Figure 5. HoxB2 inhibits neurotoxicity of mutant SOD1 in NSC-34 cells via its homeodomain. (A) Trypan blue exclusion analyses of cell death in NSC-34 cells 48 h after transfection with HoxB2, wild-type or G93A mutant SOD1 or co-transfected with G93A mutant SOD1 and HoxB2, HoxB2 homeodomain or GFP. At 48 h, 45–50% of NSC-34 cells undergo death during overexpression of G93A mutant SOD1. Co-expression of HoxB2 was associated with marked reduction of G93A mutant SOD1-induced cell death (*P < 0.05). G93A mutant SOD1-induced cell death is also significantly suppressed by co-expression of HoxB2 homeodomain. Overexpression of GFP has no effects on mutant SOD1 toxicity. (B) Similar experiment shows that co-expression of HoxB2 or HoxB2 homeodomain suppresses G86R mutant SOD1-induced neuronal cell death (*P < 0.05). (C) Trypan blue exclusion analyses of cell death in NSC-34 cells transfected with or without HoxB2 for 24 h, followed by incubation with 100 or 500 nM of staurosporine (STS) for 6 h. Staurosporine treatment of HoxB2-transfected NSC-34 cells induced 32 and 41% of cell death at 100 and 500 nM, respectively, when compared with 35 and 43% of cell death at 100 and 500 nM, respectively, for mock-transfected NSC-34 cells. No significant difference was found between HoxB2- and mock-transfected cells.
NEDL1 (39), CHIP (40,41), ALS2 (42) and Bcl2 (43). Most of the identified proteins are widely expressed, so that additional factors or interactions would be required to account for the specific vulnerability of motor neurons. It is also possible that multiple interactive targets contribute to the neurotoxicity of mutant SOD1 protein. As expression of HoxB2 is restricted to large neurons and is highly expressed in motor neurons of adult human and mouse spinal cord, interactions of HoxB2 may begin to address the specificity issue as to why only motor neurons are vulnerable to the neurotoxic effects of mutant SOD1 protein aggregates. We also show that mutant SOD1 monomers and homodimer, as well as wild-type/mutant SOD1 heterodimer, interact with HoxB2, suggesting that any of these interactions may be relevant to the dominant toxic effects of mutant SOD1 protein aggregates. Presumably, it is unclear whether neurotoxic effects of mutant SOD1 protein is conferred by their monomeric or dimeric assembly, as soluble or insoluble aggregates, or by their interactions with wild-type SOD1 forms (44).

It is also possible that interactions with mutant SOD1 may have adverse effects on the role of HoxB2 in motor neurons. This alternative view is supported by the redistribution of HoxB2 from a nuclear to cytoplasmic localization in presymptomatic G93A transgenic mice, the selective redistribution in motor neurons of transgenic mice and aggregation of HoxB2 in presymptomatic and symptomatic G93A transgenic mice. The cytoplasmic redistribution could alter the functional activity of HoxB2. The ability of Hox genes to regulate expression during embryonic development occurs at the level of transcription, as transcriptional factors that target their own transcriptional activity. During hindbrain development, each rhombomere expresses a specific combination of Hox genes. Rhombomere-restricted expression of Hox genes generates a Hox code, which is also involved in specification of motor neuron identity along the AP axis, both at spinal cord and hindbrain levels (19,45–51). Several lines of evidence also implicate Hox genes in maintaining homeostasis of fully differentiated cells. Hox genes, for example, are expressed in many different adult tissues (20,21,52,53), and deregulation of specific Hox gene expression is involved in carcinogenesis and malignant progression (22,54–56). Mutations in Hox genes also account for familial diseases such as synpolydactyly (57), hand–foot–genital syndrome (58) and Charcot–Marie–Tooth disease (CMT) (59). In CMT, mutations lead to slow progressive impairment of distal axons of fully differentiated sensory and motor neurons (60).

**Figure 6.** HoxB2 is expressed in mature brain and spinal cord and interacts with mutant SOD1 in G93A transgenic mice. (A) RT–PCR analysis of HoxB2 expression in human adult spinal cord (left panel) and mouse brain at embryonic day 8.5 (E8.5), in brain stem at embryonic day 15 (E15) and postnatal day 1 (P1) and in brain stem and spinal cord of adult mice (right panel). (B) RT–PCR analysis of HoxB2 expression in NSC-34 cells transfected with wild-type, G93A or G86R mutant SOD1 (left panel) or in NSC-34 cells transfected with G93A mutant SOD1 for 0, 24, 48 and 72 h (right panel). (C) Western blot showing expression of HoxB2 in brain and spinal cord tissues from non-transgenic, G93A transgenic and wild-type transgenic mice. (D) Brain and spinal cord extracts from presymptomatic G93A transgenic mice were incubated with anti-human SOD1 and anti-HoxB2 immunoblots, showing that HoxB2 associates with mutant SOD1 in G93A transgenic mice.
Figure 7. Pathological changes of motor neurons in G93A mutant SOD1 transgenic mice are associated with altered distribution of HoxB2 and co-localization of HoxB2 in mutant SOD1 aggregates. (A–C) Immunohistochemical reactivity showing HoxB2 enrichment in motor neurons of spinal cord (A), sensory neurons of dorsal root ganglia (B) and Purkinje neurons of cerebellum (C) and preferential localization of HoxB2 in neuronal nuclei (arrows) of adult mice. (D–F) Immunohistochemical reactivity of HoxB2 in wild-type SOD1 transgenic mice showing similar nuclear distribution of HoxB2 in motor neurons (D, arrows), sensory neurons (E, arrow) and Purkinje neurons (F, arrow). (G–I) Immunohistochemical reactivity of HoxB2 in presymptomatic G93A mutant SOD1 transgenic mice showing redistribution of HoxB2 from nuclei (arrows) to perikarya in motor neurons (G, arrow), but not in sensory (H, arrow) or Purkinje neurons (I, arrow). (J–L) Immunohistochemical reactivity of HoxB2 in lumbar spinal cord from presymptomatic (J), symptomatic (K) and end-stage (L) G93A mutant SOD1 transgenic mice. HoxB2 immunopositive punctate inclusions begin to appear in the neuropil surrounding motor neurons from presymptomatic transgenic mice (J, arrowheads). There is an increase in the intensity and number of punctate inclusions (K, solid arrowheads) and linear profiles (K, open arrowheads) containing HoxB2 in vacuolated neuropil surrounding motor neurons in symptomatic transgenic mice. At end stage, the HoxB2-positive punctate inclusions (L, solid arrowheads) and linear processes (L, open arrowheads) are readily apparent because of enhanced vacuolization of neuropil and loss of motor neurons. (M–O) Confocal microscopy showing co-localization of anti-HoxB2 (green), anti-human SOD1 (red) and merged image (yellow) of respective immunoreactivities in motor neurons and punctate inclusions in neuropil of lumbar spinal cord from G93A mutant SOD1 transgenic mouse. Scale bars; 20μm.
MATERIALS AND METHODS

DNA constructs, reagents and animals

Full-length human SOD1 cDNA was amplified by PCR from a human cDNA library and cloned into pBD–GAL4 Cam (Stratagene) and pEGFP–C1 (BD Clontech) separately. SOD1 dimers were constructed as follows: SOD1 cDNA was cloned into pMAL–c2x (New England BioLabs) at Eco RI/Bam HI and Sal I/Pst I sites of pMAL-c2x containing two copies of SOD1. Full-length human HoxB2 cDNA was purchased from GeneCopoeia, Inc (Maryland, USA) and cloned into pReceiver-M01 (GeneCopoeia), a vector with N-terminal His6-tag. Mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing. Anti-GFP, anti-human SOD1 and anti-HoxB2 were from Santa Cruz Biotechnology. Anti-His6 and Lumi-Light PLUS western blot, and immunohistochemical reactions was assessed by Sigma. Specificity of anti-HoxB2 was assessed by western blot analysis. For insolubility analysis, cells were lysed in cold lysis buffer [50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 1.5 mM MgCl2, 1% Triton X-100, 10 mM imidazole, 1 mM PMSF and mammalian protease inhibitor cocktail (Sigma)] and centrifuged at 10 000 g for 10 min at 4°C. Cell lysates were incubated with Ni-NTA agarose for 3 h at 4°C, followed by four washes with lysis buffer containing 10 mM imidazole. The Ni-NTA precipitates were subjected to standard gel electrophoresis and western blot analysis using anti-GFP and anti-His6 antibodies. For co-immunoprecipitation, brain and spinal cords from presymptomatic G93A SOD1 transgenic mice were homogenized in ice-cold homogenization buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and a cocktail of protease inhibitors using a Teflon-on-glass homogenizer and centrifuged at 10 000g at 4°C for 15 min. A total of 500 μg of protein from the supernatants was precleared with protein A/G agarose beads and incubated overnight at 4°C with anti-human SOD1 antibody. The immunoprecipitates were washed with lysis buffer and subjected to western blot analysis. For insolubility analysis, cells were lysed in phosphate-buffered saline, pH 8.0, containing 0.5% Nonidet P-40, 0.2% digitonin and 0.23 mM PMSF (lysis buffer) and centrifuged at 10 000g for 15 min to obtain detergent-soluble supernatants and detergent-insoluble pellets. Pellets were washed three times in lysis buffer and then boiled in Laemmli sample buffer. Similar amounts of supernatant and pellet fractions were analyzed by western blot analysis.

Yeast two-hybrid screen

Yeast two-hybrid screen was carried out as described previously (61). Briefly, the YRG-2 yeast strain was subsequently transformed with pBD–GAL4 Cam/G93A SOD1 (monomer or homodimer) and mouse embryonic day 12.5 spinal cord cDNA library constructed in pAD–GAL4 Cam. Colonies were selected on media lacking leucine, tryptophan and histidine, and positive clones were analyzed by β-galactosidase. Plasmid DNA was retrieved from positive clones and sequenced. On the basis of cDNA sequences identified from the yeast two-hybrid screen, full-length amino acid sequence of mouse HoxB2 was 81% identical to that of the human homolog.

RT–PCR

Total RNA was isolated from the mouse tissues with RNAeasy Mini Kit (Qiagen) and treated with RNase-free DNase I for 20 min. First-strand cDNA was synthesized using SuperScript™ First-Strand Synthesis System for RT–PCR (Invitrogen). Target cDNA was amplified with Expand High-Fidelity PCR System (Roche Applied Science) and analyzed on agarose gel.

Cell culture, transfection and cell death assay

NSC-34 cells (kindly provided by Dr Neil Cashman, University of Toronto, Canada) and Neuro 2a cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% of fetal bovine serum. Cells were transfected using FuGene 6 reagent (Roche Applied Science) following the manufacturer’s instructions. For staurosporine treatment, 24 h after transfection, cells were incubated with 100 or 500 nM staurosporine for 6 h. Cell mortality was measured by Trypan blue exclusion assay. Cell viability was expressed as the number of dead cells divided by total number of dead and viable cells.

Ni-NTA precipitation, co-immunoprecipitation, insolubility and western blot

Forty-eight hours after transfection with His6-tagged HoxB2 and various GFP-tagged wild-type or mutant SOD1, cells were lysed in cold lysis buffer [50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 1.5 mM MgCl2, 1% Triton X-100, 10 mM imidazole, 1 mM PMSF and mammalian protease inhibitor cocktail (Sigma)] and centrifuged at 10 000 g for 10 min at 4°C. Cell lysates were incubated with Ni-NTA agarose for 3 h at 4°C, followed by four washes with lysis buffer containing 10 mM imidazole. The Ni-NTA precipitates were subjected to standard gel electrophoresis and western blot analysis using anti-GFP and anti-His6 antibodies. For co-immunoprecipitation, brain and spinal cords from presymptomatic G93A SOD1 transgenic mice were homogenized in ice-cold homogenization buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and a cocktail of protease inhibitors using a Teflon-on-glass homogenizer and centrifuged at 10 000g at 4°C for 15 min. A total of 500 μg of protein from the supernatants was precleared with protein A/G agarose beads and incubated overnight at 4°C with anti-human SOD1 antibody. The immunoprecipitates were washed with lysis buffer and subjected to western blot analysis. For insolubility analysis, cells were lysed in phosphate-buffered saline, pH 8.0, containing 0.5% Nonidet P-40, 0.2% digitonin and 0.23 mM PMSF (lysis buffer) and centrifuged at 10 000g for 15 min to obtain detergent-soluble supernatants and detergent-insoluble pellets. Pellets were washed three times in lysis buffer and then boiled in Laemmli sample buffer. Similar amounts of supernatant and pellet fractions were analyzed by western blot analysis.

Immunofluorescence and immunohistochemistry

Neuro 2a and NSC-34 cells plated on round glass cover slips were fixed with methanol/aceton (1:1) for 5 min at RT. Fixed
cells were incubated with anti-HoxB2 overnight at 4°C, followed by incubation with Alexa Fluor 594 anti-goat IgG (Molecular Probes) as secondary antibody. After mounting, cells were examined by laser-scanning confocal microscopy (Zeiss LSM 510). For immunohistochemistry, paraffin-embedded human and mouse (non-transgenic, wild-type and G93A mutant SOD1 transgenic mice) sections were deparaffinized, rehydrated and antigen-retrieved in Antigen Unmasking Solution (Vector Laboratories) and then treated with 3% H2O2 to block endogenous peroxidase. After incubation with normal rabbit serum (1:10 in PBS), the sections were incubated with primary antibodies [goat polyclonal anti-HoxB2 antibody (1:100) for human spinal cord sections and rabbit polyclonal anti-HoxB2 antibody (1:100) for mouse spinal cord sections] overnight at 4°C. For light microscopy, antibody binding was visualized by the avidin–biotin–immunoperoxidase complex (ABC) method using a VECTASTAIN Elite ABC kit (Vector Laboratories) following the manufacturer’s recommendations and DAB Substrate kit (Vector Laboratories). For confocal microscopy, after incubation with primary antibody, sections were incubated with biotinylated secondary antibody, followed by incubation with Fluorescein Avidin D or Texas Red Avidin D.

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Conflict of Interest statement. None declared.

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