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Fine Mapping of an Amyotrophic Lateral Sclerosis Modifier Locus to a Single BAC

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Abstract

Mutations in the Cu/Zn superoxide dismutase (SOD1) gene are responsible for 15-20% of familial amyotrophic lateral sclerosis (FALS) cases. Severity of the ALS phenotype is dependent on genetic background for mice carrying the mG86R SOD1 transgene. A BAC (149m19) containing several candidate genes (Serf, Smn, Naip2 and Naip5) and derived from 129Sv genomic DNA was used to investigate whether these genes partially rescue the mG86R SOD1 (FVB/NJ) ALS phenotype by delaying ALS onset. Two single-insertion BAC lines were produced and both mRNA and protein for BAC genes were shown to be elevated. Mice heterozygous for mG86R SOD1 and BAC149m19 in the FVB/NJ background were shown to have significantly delayed ALS onset compared to mG86R SOD1 controls. BAC 149m19 did not significantly delay ALS onset for mice carrying the human G93A SOD1 mutation in the FVB/NJ background. Delayed ALS onset for BAC/mG86R mice was not due to reduced SOD1 expression. Overexpression of one or more of the BAC genes appears to be linked to the severity of ALS phenotype in mice, but in a mutation specific manner.

Introduction

Amyotrophic lateral sclerosis is a progressive motor neuron disease (MND) leading to death for diagnosed patients, usually within 2 to 5 years, primarily due to respiratory failure. ALS has no known cure and limited therapeutic options. Most cases of ALS are sporadic, with only 5-10% being inherited. Of those familial cases (FALS), 15-20% possess a mutation in the cytoplasmic Cu/Zn superoxide dismutase (SOD1) gene. To date, there are about 114 different mutations in the SOD1 gene that are associated with FALS cases [1]. Current research supports the idea that SOD1-linked ALS may be caused by misfolded protein and aggregates. Thus, SOD1-linked ALS is thought to involve a gain of (toxic) function phenomenon that is independent of enzymatic activity.

In people, ALS can vary greatly in onset and progression, suggesting that genetic modifiers may be influencing aspects of the disease [2,3]. Like ALS in humans, there is also significant variation in ALS phenotype (onset, duration, and certain histopathological characteristics) in SOD1 transgenic mice [4]. Identification of the genetic factors associated with such variation may prove useful in providing additional targets for treatment therapies.

In 2000, Kunst et al. reported on their observations on the lifespan of transgenic mice carrying the mG86R SOD1 mutation, which is analogous to the hG85R SOD1 mutation in humans. The mG86R SOD1 mice in the FVB/NJ background succumb to ALS at about 106 days. However, two heterozygotes from a cross between mG86R SOD1 (FVB/NJ) and C57B16/129Sv lived for more than a year before developing ALS symptoms. It was hypothesized that the differences in the genetic backgrounds of the two strains were most likely responsible for the delayed development of ALS symptoms. A subsequent linkage analysis showed that the best evidence for linkage of the delayed ALS onset modifier genes was observed on mouse chromosome 13 between D13Mit36 and D13Mit76.The above mentioned region contains a number of promising candidate genes including survival of motor neuron (Smn), multiple copies of neuronal apoptosis inhibitory protein (Naip), and small EDRK-rich factor (Serf). Deletions of SMN are associated with human spinal muscular atrophy or SMA [5,6]. SERF is thought to modify spinal muscular atrophy [5]. Deletions of NAIP are believed to modulate the severity of SMA [7]. This region is represented in a BAC originally isolated by Endrizzi et al. [6] from a mouse library constructed by 129Sv genomic DNA (see Figure 1). Mouse versions of Serf and Smn are present on the BAC as are two different murine Naip genes. To investigate whether these BAC genes rescue or partially rescue the G86R SOD1 phenotype by delaying ALS onset, BAC149m19 transgenic mice were made and crossed with mG86R SOD1 (and hG93A SOD1 for comparison) transgenic mice. The age at ALS onset and survival in these heterozygotes were determined. Identification of genes that suppress the development of ALS may help to clarify the mechanism by which mutations in SOD1 lead to ALS.

Methods

Animals: All mouse experiments were approved by the University of Denver Institutional Animal Care and Use Committee. The mG86R SOD1 mice were obtained from John Gordon (Mount Sinai School of Medicine, New York) and later deposited in The Jackson Laboratory (Strain Name FVB-Tg(Sod1*G86R)M1Jwg/J; Stock Number: 005110). The G93A SOD1 mice were obtained from The Jackson Laboratory (Strain Name: B6SJL-Tg(SOD1-G93A)1Gur/J, Stock Number 002726).

All mice were maintained on an FVB/NJ background (Jax). The G93A SOD1 mutation was transferred to the FVB/NJ background by back crossing the male G93A SOD1 mice to female FVB/NJ until the 129 polymorphic markers that were assayed [see 4] were of FVB/NJ origin including D12mit239 which had been segregating with the G93A SOD1 transgene. Mice carrying an SOD1 mutation were observed daily for ALS onset, which was defined as when mice exhibited defects in gait or splay. When mice placed on their sides could no longer right themselves after 10 seconds, they were humanely euthanized by cervical dislocation. Time at euthanasia was used for survival analysis. Dissected tissues were rinsed in PBS to remove blood prior to freezing in Liquid Nitrogen. All tissues were stored at -80oC prior to use.

Creation of 149m19 BAC transgenic mice: The 149m19 BAC (Genbank AF131205) was a gift from Dr. William F. Dietrich, Department of Genetics, Harvard Medical School. BAC DNA was purified with the Nucleobond AX kit (Clontech). The 149m19 BAC transgenic mice were created by injecting purified Not I or BsiWI (New England Biolabs) digested BAC DNA, for line 1199 and line M141 respectively, into FVB/NJ fertilized oocytes using standard methods [27]. Not I digestion removes the pBeloBAC11 sequence while BsiWI digestion linearized the BAC. Transgenic founders were detected with 11 polymorphic primer sets (see Table 1) that differentiate between 129Sv and FVB/NJ.

Fluorescence in Situ Hybridization (FISH): Florescence in situ hybridization was performed as described previously [27] in order to ensure that the 149m19 BAC had a single insertion site in each line. A portion of mouse chromosome 16 was SpectrumOrange-labeled and used as a control probe (gift from Dr. David Patterson, University of Denver). SpectrumGreen-labeled 149m19 test probe was prepared as described in the Nick Translation Kit (Vysis). Blood smears were prepared from each of the transgenic pups on pre-cleaned Superfrost/Plus microscope slides (Fisher). The smears were fixed for 2 minutes in methanol:glacial acetic acid (1:1 vol:vol) and 2 minutes in methanol:glacial acetic acid (3:1 vol:vol). Slides were fixed for 2 minutes in 1:1 (v/v) and then for 2 minutes in 3:1 (v/v) methanol:glacial acetic acid. Five µl of each probe were placed onto each slide, covered with 18mm2 cover slip, and denatured by placing them on an 80ºC heat block in the dark for 5 minutes. Slides were allowed to hybridize overnight in a humidified 37ºC chamber. Slides were post-washed in 0.4× SSC at 72 ± 1 °C for 1 min, then in 2× SSC/0.1% NP-40 at room temperature for 1 min. Slides were allowed to dry, then counterstained with API (4′,6-diamidino-2-phenylindole) / Antifade (Vysis) and stored at 4°C. Hybridized nuclei were visualized using a Zeiss Axiovert 200M Microscope with DAPI and Cy3 filters (to detect DAPI counterstain and Spectrum Orange-labeled P33A12 probe, respectively). Representative nuclei were analyzed with a Zeiss Axiovert 200 inverted epifluorescence microscope with Zeiss Axiovision image analysis software.

Crosses and Genotyping: Transgenic BAC149m19 females were crossed with either mG86R SOD1 or G93A SOD1 males. The Gentra systems DNA preparation kit was used to purify genomic DNA from 2 mm tail biopsies per the manufacturer's instructions. MIT polymorphic primer sets were obtained from Research Genetics and used as described previously [4]. D13CBK5 was used to identify the 149m19 transgene. Multiplex PCR was used to genotype the mG86R SOD1 construct. The forward and reverse primers target regions of the murine SOD1 gene, and the third primer specifically recognizes the mG86R SOD1 mutation in the transgene. Thus, the mG86R SOD1 mice are identified by the appearance of two bands on the gel. Multiplex PCR was used to genotype transgenic mice containing the G93A SOD1 construct. The murine IL2 gene was amplified as an internal control. Primer sequences are given in Table 2.

Twenty-five percent of the offspring of crosses between the BAC lines with the SOD1 mutant lines, mG86R and G93A are expected to have both the BAC and a mutant form of SOD1, while another 25% are expected to have just the SOD1 mutation. The ages at onset and euthanasia were used for Kaplan-Meier survival analysis (GraphPad Prism 5).

TaqMan Real-Time Quantitative PCR Assays: TaqMan experiments were conducted on 4 mice from each line with 3 to 6 replicates per mouse. Total RNA

was prepared from a 40-50 mg piece of mouse cortex for the Smn and Serf assays or spleen for the Naip2 and Naip5 assays using the GenElute Mammalian Total RNA Kit (Sigma) as recommended. These tissues were used because of the difficulty in obtaining sufficient amounts of the most relevant tissue, i.e., spinal cord. RNA samples were stored in aliquots at -80oC until use. The RETROscript First Strand Synthesis Kit (Ambion) was used to make cDNA per the manufacturer's instructions. Final concentrations of 25ng/µl for the Smn and Serf assays and to 12.5ng/µl for the Naip2 and Naip5 assays were used. The ABI 7900HT Sequence Detection System with SDS2.1.1 Software was employed. Cycling parameters for all primer sets (10µl reactions) were 95°C for 10 minutes, 45 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. All probes and the rodent GAPDH-Vic control reagents were purchased from Applied Biosystems. Smn and Serf were multiplexed with GAPDH using the primer concentrations given in Table 2 and 60 nM GAPDH Forward Primer, 60 nM GAPDH Reverse Primer, 130 nM GAPDH-VIC Probe, using 50ng cDNA. Naip2 and Naip5 were multiplexed with GAPDH using primer concentrations in Table 2 and 50 nM GAPDH Forward and Reverse Primers, and 130nM GAPDH-VIC Probe, using 25ng cDNA. Both Naip2 and Naip5 PCR reactions included controls without reverse transcriptase to account for any background amplification from pseudogenes and/or DNA contamination, as the Naip primers did not span an intron. Primer sequences and concentrations used in quantitative PCR are given in Table 2. Statistical testing was performed by one-tailed t-tests of each transgenic line versus the FVB control using mouse averages.

Immunoblotting: Quantitative western blots were used to characterize protein expression of Smn in BAC transgenic mice. Only Smn protein was assayed since, at the time, antibodies for the other gene products were either not readily available or not specific enough. Protein was prepared from a 10 mg piece of cortex that was cut off while frozen on dry ice. Tissue was homogenized in DTT lysis buffer (9mM Urea, 100µM DTT, 4% Nonidet P40) plus one Complete Mini Protease Inhibitor Tablet (Roche), spun for 5 minutes at maximum rpm in a table top centrifuge, and the supernatant was transferred to a fresh tube. Protein concentration was measured using the Bio-Rad Bradford Protein Assay Reagent, according to manufacturer's protocol. Eight ug of protein were mixed 1:1 with 2X Novex Tris-Glycine SDS Sample Buffer (Invitrogen), boiled for 5 minutes and loaded onto 14% Novex Tris-Glycine polyacrylamide gels (Invitrogen). Proteins were separated at 15 mA per gel for 90 minutes and then transferred to nitrocellulose membranes overnight at 30 mA in a buffer of 25mM Tris, 52mM Glycine, 20% Methanol. Blots were stained with Ponceau S to verify transfer and blocked for 60 minutes at room temperature in a buffer of 5% non-fat dry milk, 1X TBS (20mM Tris, 150mM NaCl). The primary antibody was incubated with the blot in 1X TMT (0.2% Triton X-100, 1X TBS) plus 1% non-fat dry milk for 90 minutes at room temperature. Blots were washed 3 x 5 min with TMT and incubated with the secondary antibody for 1 hour in TMT. Following two 5 min washes with TMT and two 3 min washes with 1X TBS, the antibodies were detected with the ECL PLUS Chemiluminescence Kit (Amersham) and visualized on ECL Hyperfilm (Amersham). Antibody dilutions were: Smn (BD Transduction Laboratory) 1:1000, HRP linked goat anti-mouse secondary 1:10,000 Enolase 1:1000, HRP linked goat anti-rabbit secondary 1:10,000, SOD1 1:5000 (Calbiochem), and HRP linked donkey anti-sheep secondary 1:10,000 (Jackson ImmunoResearch). Autoradiographs were imaged on an EDAS 290 Electrophoresis Documentation and Analysis System (Kodak) and densities were determined using the Kodak 1D image analysis software. Eight replicates were done for FVB and line 1199 and six replicates were done for line M141. Quantities were normalized to a standard curve and ratios of SMN:Enolase and SOD1:Enolase were normalized to the FVB/NJ samples.

Results

Two different transgenic BAC lines were produced (line 1199 and M141) in order to insure that any phenotypic differences were due to the expression of BAC genes and not due to its insertion. That the BAC149m19 had a single insertion site in each line is supported by fluorescence in situ hybridization. Figure 2 shows that both of the transgenic lines had one additional hybridization of the SpectrumGreen labeled 149M19 probe representing the additional BAC149m19 sequence.

Quantitative RT-PCR was done to test the relative expression of BAC 149m19 genes in transgenic mice versus controls, and the data are shown in Figure 3. The mRNA levels for Serf, Smn, and Naip2 are all significantly elevated in the transgenic lines compared to the FVB controls, usually at the .01 statistical level. The Naip5 gene only showed significantly elevated mRNA expression in one BAC line (M141). Generally, overexpression was in the range of 1.5 to 2.5 fold. In addition, a commercially available Smn antibody was

used to verify that the 149m19 BAC transgenic mice overexpress the Smn protein. Both transgenic lines show significantly elevated Smn protein concentrations (Figure 4). Overexpression of this protein was around 2-2.5 fold and statistically significant at the P < .01 level. Because previous research on G93A SOD1 mutations in mice showed that there is a gender difference in certain strains, with females living longer [8], a test of male versus female survival time was performed for both mG86R and G93A SOD1 mutations in the FVB/NJ background. The results are shown in Figure 5. There is no difference in survival time for males and females for the mG86R SOD1 mice, which is consistent with previous observations [4] but, as has been observed before in other genetic backgrounds, survival of females carrying the G93A SOD1 mutation in the FVB/NJ background is significantly longer than males.

Since there are no gender differences, the survival results for mG86R SOD1/BAC149m19 transgenic mice are shown with both sexes combined (Figure 6). The Kaplan-Meier graph shows that median survival time of mG86R SOD1/BAC 149m19 heterozygotes is significantly longer than the mG86R SOD1 mice without the BAC, and this can be clearly seen in both BAC lines. The control mG86R SOD1 mice median survival time was 106 days while the medians for the BAC lines were 112 days and 119 days for lines M141 and 1199 respectively. Log-rank (Mantel-Cox) statistical analyses shows that the increased survival time is statistically significant in both lines (line M141: Chi Square = 6.689, df = 1, P < 0.01; and line 1199: Chi Square = 14.470, df = 1, P < 0.001).

Figures 7 and 8 (females and males respectively) show the equivalent Kaplan-Meier graphs for the G93A SOD1 mutation with sexes analyzed independently. Mantel-Cox statistics for females were Chi Square = .056, df = 1, P > 0.05 (line 1199) and Chi Square = 3.359, df=1, P > 0 .05 (line M141), while those for males were Chi Square = 0.991, df = 1, P > 0.05 (line 1199) and Chi Square = 0.325, df = 1, P > 0 .05 (line M141). Thus, regardless of gender, there are no statistically significant differences in survival time between G93A SOD1/BAC 149m19 heterozygotes and G93A SOD1 mice without the BAC.

To investigate whether the increased survival time in mG86R SOD1/BAC149m19 transgenic heterozygotes might be due to a lower expression of SOD1 protein in these animals compared to the mG86R SOD1 control animals, western blot analysis was performed. Statistical comparisons between the BAC lines and the control animals showed that there were no statistically significant differences in SOD1 protein levels between controls and either BAC line (line 1199: ts = 2.776, df = 4, P > .05; and line M141: ts = 2.447, df = 6, P > .05). We also checked to see if the disease duration (i.e., the period from disease onset to the time point at which the mice were euthanized) contributed to the BAC partial rescue. Disease duration for neither BAC line was significantly different from that of the controls (data not shown).

Discussion

Transgenic mice have proven very useful in the investigation of the mechanisms of toxicity of SOD1 mutants, even though the neurodegenerative mechanism is still not fully elucidated [see 1,9 for reviews]. Transgenic mice that express SOD1 mutations exhibit phenotypes that are similar to symptoms of human ALS patients, including weakness, tremors, and limb paralysis. Strains expressing human SOD1 missense mutations G37R, G85R, D90A, G93A, [10-16] as well as truncated L126Z and G127X SOD1 [17] have been used as ALS models. In this study, we employed a transgenic strain that overexpresses the murine G86R (mG86R) SOD1 mutation (analogous to hG85R) [18]. These transgenic strains are generally characterized by shortened life spans and mutant SOD1 mRNA levels that are significantly higher than the levels of endogenous SOD1 [1] However, transgenic mice overexpressing wildtype human SOD1 do not develop MND disease suggesting that ALS phenotype is unrelated to SOD1 expression level [10,11].

In humans, there is clinical variation among patients who are members of the same family and who carry the identical SOD1 mutation. In some cases, individuals carrying an ALS associated SOD1 mutation may not even develop the disease [19]. One specific SOD1 mutation, D90A, is recessive in some ethnic backgrounds and dominant in others [20,21]. The same variation phenomenon is observed in SOD1 transgenic mice. In some genetic backgrounds (ALR, NOD.Rag1KO, SJL or C3H) the time to G93A SOD1-induced ALS onset is shortened, and thus, a more severe phenotype is observed. In other backgrounds (B6, B10, BALB/c and DBA), a milder phenotype is observed since the time to disease onset is lengthened [8]. For this mutation, there is a distinct sexual dimorphism with respect to survival time in some lines where females live longer than males [8].

The existence of BAC149m19 has provided a unique opportunity to investigate the effect of several candidate genes on the ALS phenotype that is expressed by mG86R SOD1 FVB/NJ transgenic mice. This genetic locus is quite divergent between mice and

men. Mice have a single Smn gene and 5 to 7 functional Naip homologs [22,23] while humans have two SMN genes and a single NAIP gene. All of the BAC genes (Serf, Smn, and Naip) have been associated with SMA, which like ALS, is a disease that is characterized by the loss of lower motor neurons. Although, mutations in SMN and/or NAIP have not been associated with ALS [24], polymorphisms within or expression levels of these genes might modulate the toxicity of mutant SOD1. Indeed, abnormal copy number of SMN1 appears to be a susceptibility factor for sporadic ALS [25,26]. It is reasonable then to ask: do the BAC genes mimic the delayed onset of ALS observed when this SOD1 mutation is placed in a mixed background of C57B16/129Sv?

BAC 149m19 transgenic mice with additional copies of strain 129Sv derived Serf, Smn, and Naip genes were expected to have elevated gene expression and gene product production. The data presented in Figures 3 and 4 substantiate this expectation. Thus, it appears that BAC genes are being expressed and, for at least one gene, this translates into increased protein expression.

The Kaplan-Meier survival curves shown in Figures 6-8 support the contention that one or more of the genes present on the BAC has a significant effect on the ALS phenotype of the mG86R but not the hG93A SOD1 mutation. Sexes for the hG93A SOD1 mutation were analyzed separately since the significant gender differences shown in Figure 5 for this mutation make gender-specific analysis a necessity. The effect of the 129Sv derived BAC genes on ALS phenotype appears to be mutation or species specific. Our results also indicate that SOD1 protein levels are not significantly different between the BAC lines and the control animals. Thus, reduced expression of mutant SOD1 protein does not explain the partial BAC rescue phenomenon. Since the BAC lines and the controls are not significantly different with respect to disease duration, the majority of the partial rescue effect of the BAC appears to be due to its effect on ALS onset.

The fact that ALS phenotype is dependent on the genetic background is very important in studies of ALS using mouse models. First, it mimics the situation of background dependent ALS in humans. Obviously then, care must be taken in studies using mice to insure that the same genetic background is used for both experimental and control animals so that differences can be attributed to the phenomenon being investigated and not to differences in genetic background [8].

The 129Sv derived 149m19 BAC modifier genes (Serf, Smn, Naip2 or Naip5) appear to act in either a mutation (mG86R vs. G93A) or species (murine vs. human transgene) specific manner. We hypothesize that variations in DNA sequence between the 129Sv and FVB/NJ strains are responsible for the protective effects of the 149m19 BAC genes on the mG86R SOD1 mouse model of ALS. We do not yet know which gene or gene combination is responsible for the effect. They all remain excellent candidate modifier genes due to their association with both SMA and ALS in people. Thus far, no exonic variation has been found in the Serf and Smn genes. Analyses of Naip2 and Naip5 are ongoing. Future experiments will involve mutating each gene in 149m19 BAC to make single gene knock-out constructs and then testing whether they produce the delayed ALS onset observed with the unmutated BAC. We are also very interested in the histopathology of the BAC-mediated partial rescue. It would be informative to determine whether the G86R/BAC heterozygotes have more motor neurons, a slower rate of motor neuron loss, or less severe inflammatory changes within the spinal cord than the G86R SOD1 strain. These experiments are planned or are in progress. Finally, it is unclear why the BAC genes would protect against mG86R SOD1 but not hG93A SOD1 mediated ALS. One possibility is that the 149m19 BAC genes would be protective for the G93A SOD1 mutation if that mutation were carried on a murine, rather than human, form of the gene.

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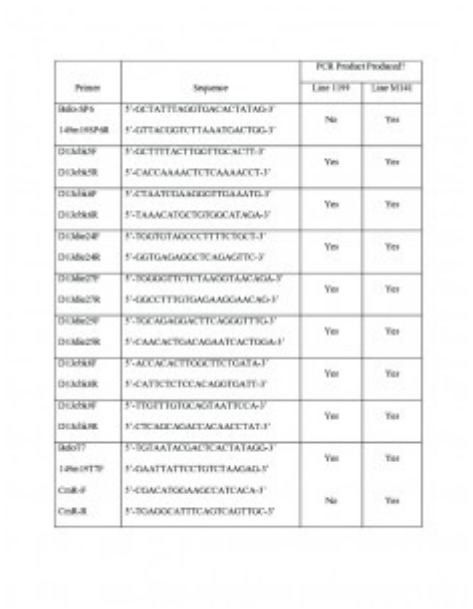
Illustrations

Illustration 1

Table 1. Primer sequences used to identify BAC149m19 transgenic founders.



Primer	Sequence	PCR Product Produced?	
		Line 1399	Line 9144
Hdlx-SP6	5'-GCTATTTAGGTGACACTATAG-3'	No	Yes
149m19SP6R	5'-GTTACGGTCTTAAATGACTGG-3'		
D13cf63F	5'-GCTTTTACTTGGTTGCACTT-3'	Yes	Yes
D13cf63R	5'-CACCAAAACTCTCAAAACCT-3'		
D13cf64F	5'-CTAATTGTAAGGGTTGAAATG-3'	Yes	Yes
D13cf64R	5'-TAAACATGCTGTGGCATAGA-3'		
D13dn24F	5'-TGGTGTAGCCCTTTTCTCCT-3'	Yes	Yes
D13dn24R	5'-GGTGAGAGGCTCAGAGTTC-3'		
D13dn27F	5'-TGGGGTTCTCTAAGGTAACAGC-3'	Yes	Yes
D13dn27R	5'-GGCCTTGTGAGAAGGAACAG-3'		
D13dn29F	5'-TGCAGAGGACTTCAGGGTTG-3'	Yes	Yes
D13dn29R	5'-CAACACTGACAGAATCACTGGA-3'		
D13cf68F	5'-ACCACACTTGGCTTCTGATA-3'	Yes	Yes
D13cf68R	5'-CATTCTCTCCACAGGTGATT-3'		
D13cf69F	5'-TTGTTTGTGCAGTAATTCCA-3'	Yes	Yes
D13cf69R	5'-CTCAGCAGACTCACAACTCTAT-3'		
Hdlx57	5'-TGTAATACGACTCACTATAGG-3'	Yes	Yes
149m19T7P	5'-GAATTATTCCTGTCTAAGAG-3'		
Cre8-F	5'-CGACATGGAAGCCATCACA-3'	No	Yes
Cre8-R	5'-TGAGGCATTTCAGTCAGTTGC-3'		

Illustration 2

Table 2. Primer Sequences

Primer	Sequence	Conc.
Quantitative PCR		
TM SMN F	5'-GAATGCCACAACTCCCTTGAA-3'	60nM
TM SMNR2	5'-CAATTGGACGTAATACGTAGCTTGGCTAA-3'	60nM
6FAM SMN Probe	5'-AAAGTTGCTGACAAGTGTTCTGCTG-3'	130nM
SERF1 6F	5'-CGAAAAAGAAAAGAAGCACAGCTTGA-3'	30nM
SERF1 6R	5'-CATAGATTTCTTCTCATTGGCTATCTTC-3'	90nM
6FAM SERF Probe	5'-AGAGGGATTCAGAGATCATCCAACAAAAG-3'	130nM
TM Naip2F	5'-TGTCAGGGTTATTTTATCCCAGCAA-3'	200nM
TM Naip2R	5'-AGACAGCTCACAGACCAAGTCGTCAT-3'	200nM
6FAM Naip2 Probe	5'-CAGCCCAGGGAGAAGCCGTTGA-3'	130nM
TM Naip1 UTR-F	5'-AAAGCGCGTTGGGCCCTCCT-3'	30nM
TM Naip1 UTR-R	5'-GCCTTCCGGATAGATGGATCA-3'	80nM
6FAM Naip1-UTR Probe	5'-CCATTTGGGTGCTGGTCACCAAA-3'	130nM
Genotyping		
Mutant SOD1-F	5'-TGTTCATCCAACCTGATGCTG-3'	
Mutant SOD1-R	5'-CCAATGATGGAATGCTCTCC-3'	
mG93R SOD1-R	5'-TAGGCATGTTGGAGACCTGC-3'	
hG93A SOD1-F	5'-CATCAGCCCTAATCCATCTGA-3'	
hG93A SOD1-R	5'-CGCTACTAACAATCAAAGTGA-3'	
murine IL2-F	5'-CTAGGCCACAGAATTGAAAGATCT-3'	
murine IL2-R	5'-GTAGGTGGAAATTCTAGCATCATCC-3'	

Illustration 3

Figure 1. Schematic representation of BAC149m19 showing locations of candidate genes (in bp). Genes marked with an * are present in line 1199 and those marked with a # are present in line M141.

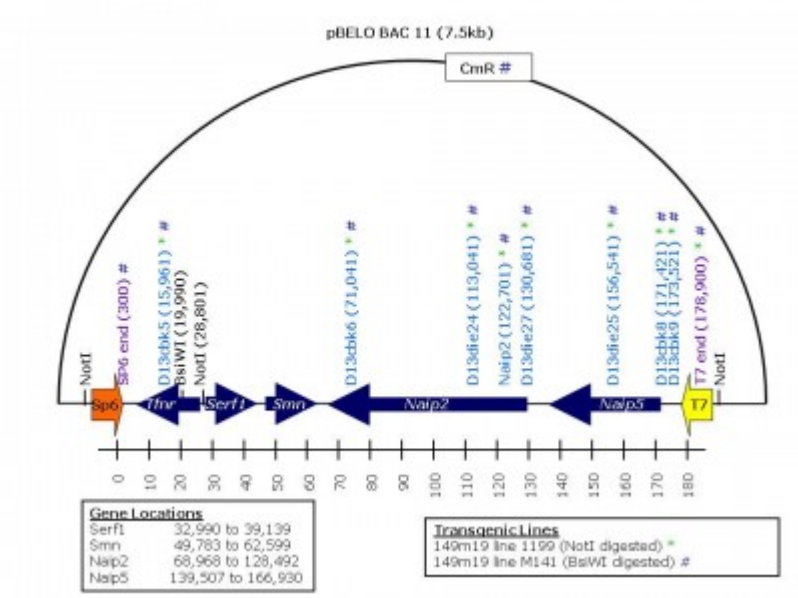


Illustration 4

Figure 2. Representative images of fluorescence in situ hybridization. Red = SpectrumOrange-labeled chromosome 16 sequence control probe. Green = SpectrumGreen-labeled 149m19 test probe. Both transgenic lines have one extra green spot compared to non-transgenic mice.

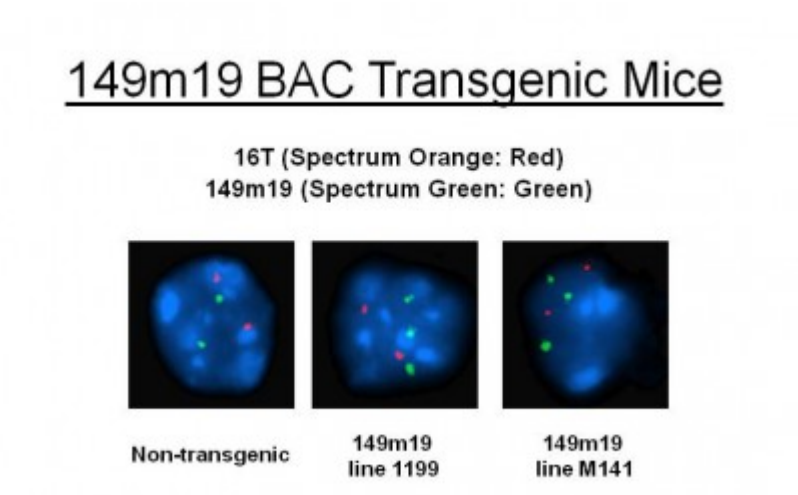


Illustration 5

Figure 3. Relative expression of 149M19 BAC genes in transgenic mice (lines 1199 and M141) vs. control (FVB). All mRNA experiments were normalized to GAPDH expression. Gene/GAPDH ratios were then normalized to the FVB average for each gene. Bars represent the averages of 4 mice from each line /- the standard error (se). Asterisks indicate significance level in one-tailed t-tests of each transgenic line vs. control (*P < .05; **P < .01).

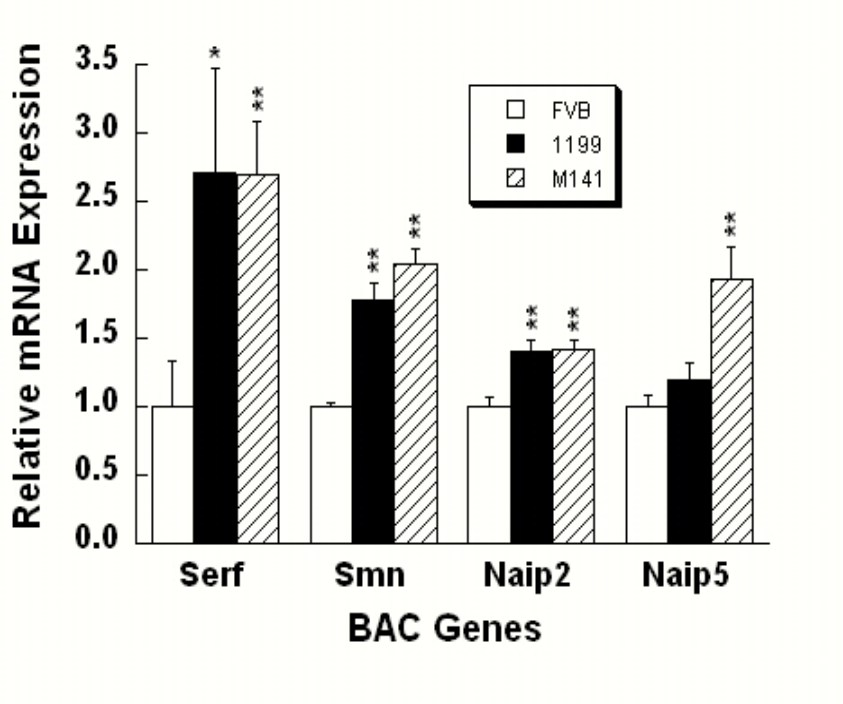


Illustration 6

Figure 4. Relative expression of Smn protein in transgenic mice (lines 1199 and M141) vs. controls (FVB). Concentrations were derived from a standard curve. Smn/enolaseratios were then normalized to the FVB average. Bars represent the averages of multiple replicates +/- the standard error (se). Asterisks indicate significance level in one-tailed t-tests of each transgenic line (**P < .01).

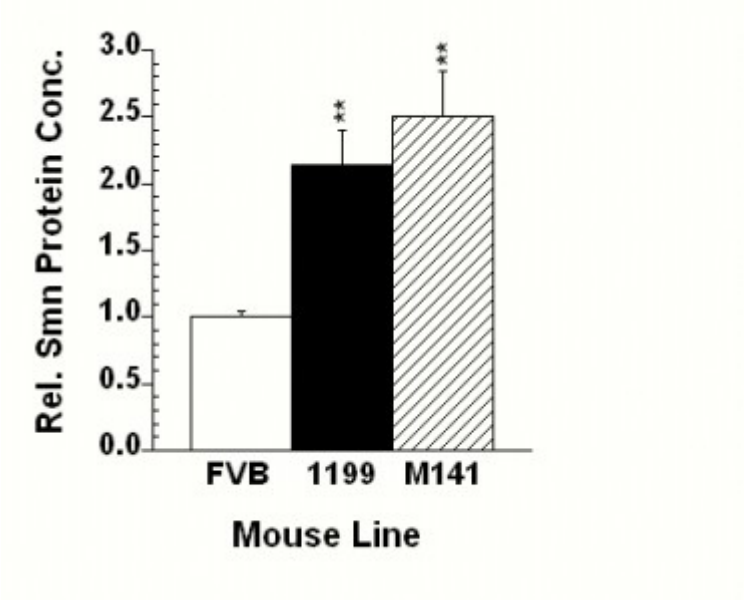


Illustration 7

Figure 5. Gender differences in average survival (in days) for mG86R and G93A SOD1 transgenic mice Â± standard error (se). There is no statistically significant gender difference for G86R SOD1 mice (ts = 0.296, df = 113, P > .05), but the gender difference for G93A SOD1 mice is highly significant (ts = 3.719, df = 128, P < .001).

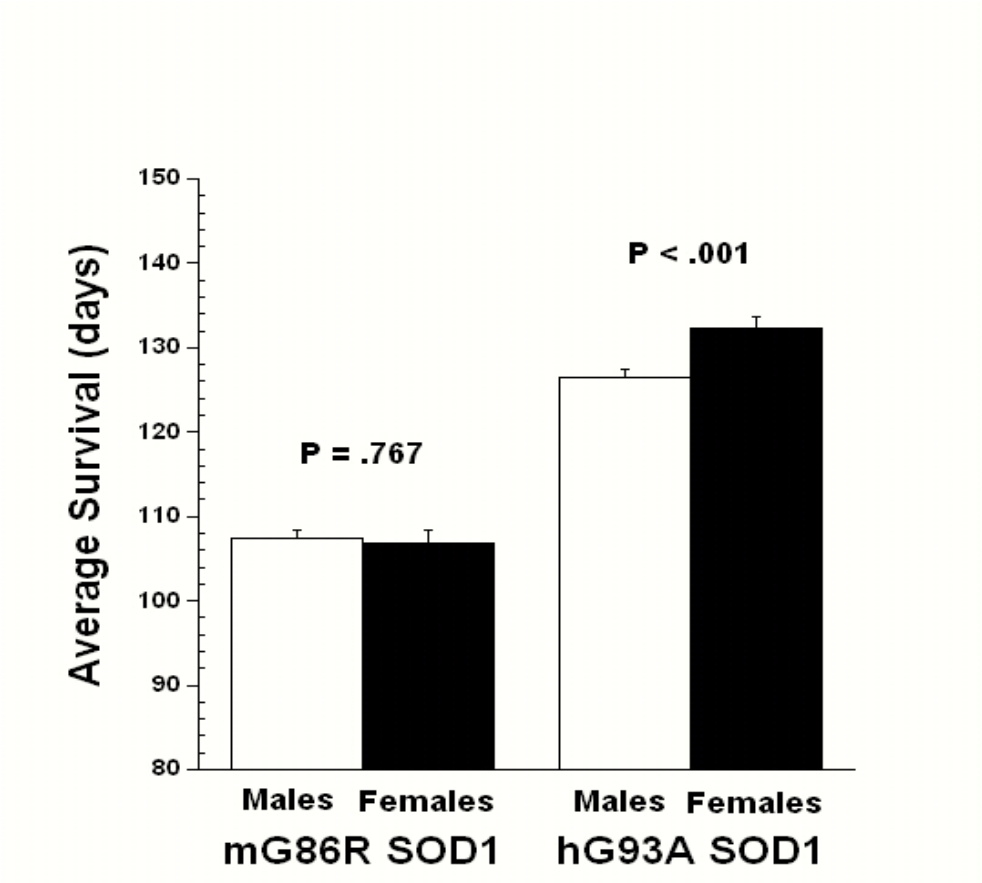


Illustration 8

Figure 6. Kaplan-Meier graph showing the effects of BAC 149m19 (lines 1199 and M141) on the survival of mG86R SOD1 mice (sexes combined). Vertical bars represent standard errors. mG86R SOD1/BAC lines were statistically compared to the mG86R SOD1 controls using a log-rank (Mantel-Cox) test. Survival is significantly increased in both line M141 (Chi Square = 6.689, df = 1, P < .01) and line 1199 (Chi Square = 14.470, df = 1, P < .001).

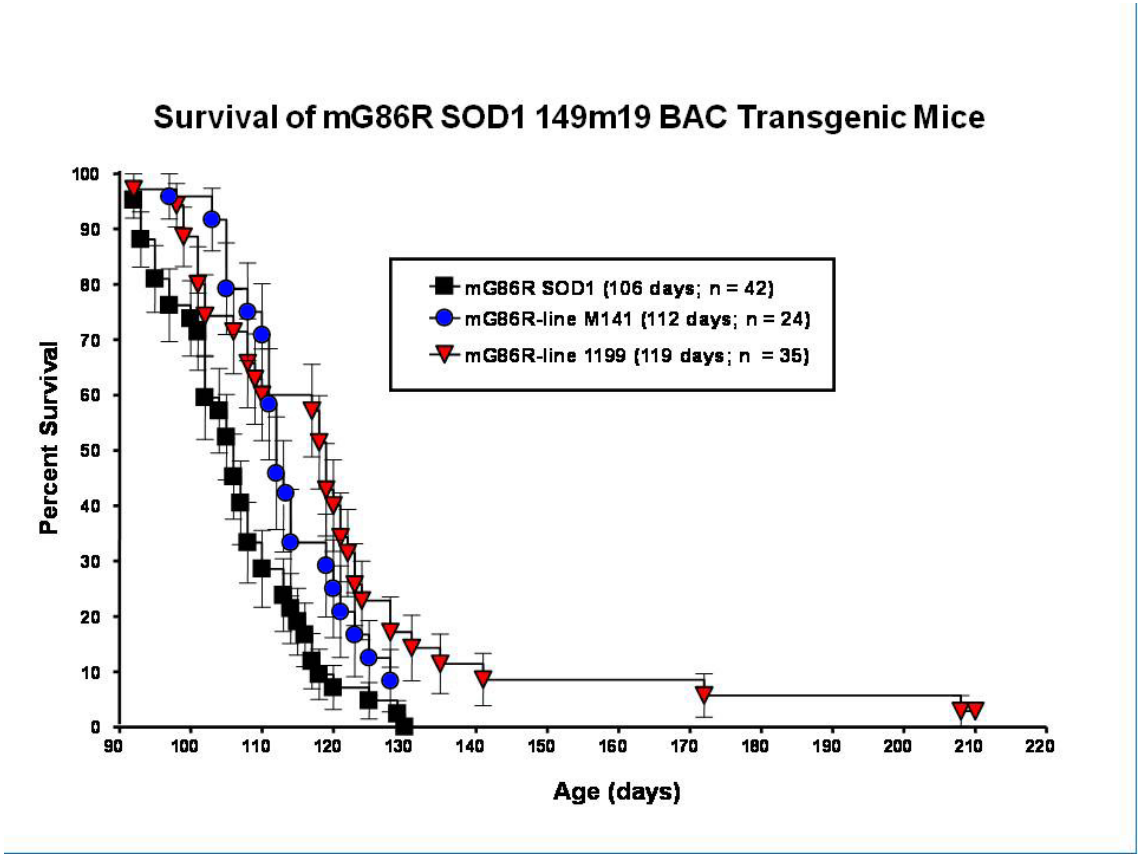


Illustration 9

Figure 7. Kaplan-Meier graph showing the effect of BAC 149m19 on the survival of G93A SOD1 female mice. Vertical bars represent standard errors. Curves were statistically compared using log-rank (Mantel-Cox) tests. The results show that the presence of the BAC in females has no significant effect on survival (line 1199 Chi Square = .056, df = 1, P > 0.05; line M141 Chi Square = 3.359, df=1, P > 0 .05).

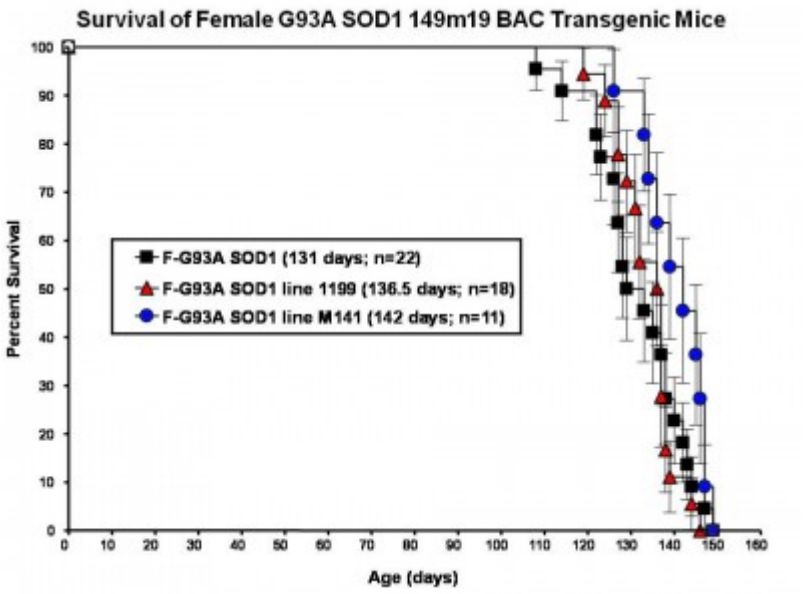
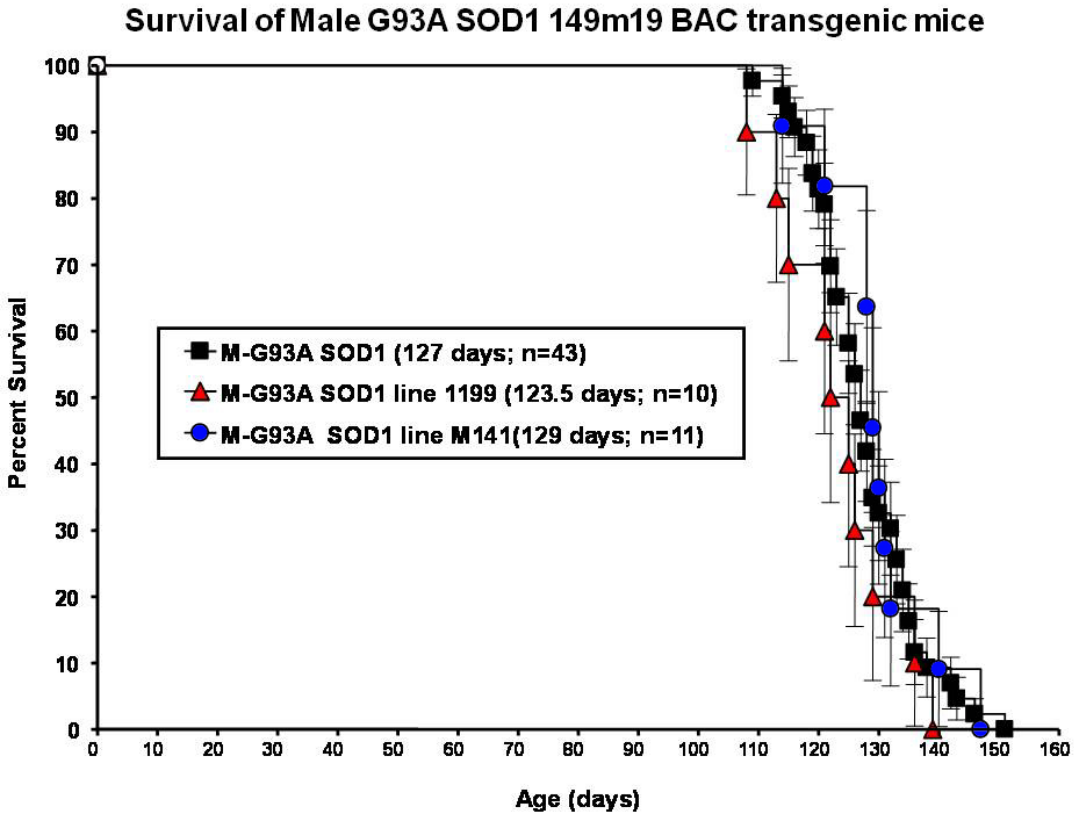


Illustration 10

Figure 8. Kaplan-Meier graph showing the effect of BAC 149m19 on the survival of G93A SOD1 male mice. Vertical bars represent standard errors. Curves were statistically compared using log-rank (Mantel-Cox) tests. The results show that the presence of the BAC in males has no significant effect on survival (line 1199 Chi Square = 0.991, df = 1, P > 0.05; line M141 Chi Square = 0.325, df = 1, P > 0 .05).



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