Gene Targeting of Mouse Tardbp Negatively Affects Masp2 Expression

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating adult onset neurodegenerative disease affecting both upper and lower motor neurons. TDP-43, encoded by the TARDBP gene, was identified as a component of motor neuron cytoplasmic inclusions in both familial and sporadic ALS and has become a pathological signature of the disease. TDP-43 is a nuclear protein involved in RNA metabolism, however in ALS, TDP-43 is mislocalized to the cytoplasm of affected motor neurons, suggesting that disease might be caused by TDP-43 loss of function. To investigate this hypothesis, we attempted to generate a mouse conditional knockout of the Tardbp gene using the classical Cre-loxP technology. Even though heterozygote mice for the targeted allele were successfully generated, we were unable to obtain homozygotes. Here we show that although the targeting vector was specifically designed to not overlap with Tardbp adjacent genes, the homologous recombination event affected the expression of a downstream gene, Masp2. This may explain the inability to obtain homozygote mice with targeted Tardbp.

Materials and Methods

Generation of Tardbp targeting vector

To generate a conditional knockout of the Tardbp gene a mouse bacterial artificial chromosome (BAC) clone containing Tardbp (RP23-29102) was obtained from the Children's Hospital Oakland Research Institute (CHORI, https://bapac.chori.org/) and modified by recombineering. First, RP23-29102 was modified for recombination by electroporation of PSC101gbaA plasmid encoding the recombination machinery. Following integration of a Zeo cassette in intron 1 of the Tardbp gene, a loxP site was exchanged with the Zeo cassette and placed as the most 5' loxP recombination site, upstream of exon 2. A neomycin resistance cassette flanked by FLP recognition target (FRT) sites was amplified by Polymerase Chain Reaction (PCR) and integrated downstream of Tardbp exon 3. Finally, the modified gene was transferred to a plasmid containing the diphtheria toxin cassette to generate the targeting construct (Figure 1).
Screening of ES clones and mouse genotyping

The Tardbp targeting vector was electroporated into C57BL6/129 embryonic stem (ES) cells at the Toronto Centre for Phenogenomics (http://www.phenogenomics.ca/). Initial identification of positive ES cell clones was performed by ethanol precipitation of genomic DNA (gDNA) and PCR amplification using primers specific for the most 5' loxP site. We found 8 positive ES clones which were subsequently expanded in 24 well plates and screened for recombination of the 5' and 3' homology arms by sequencing and Southern blot analyses, respectively. The sequence of the 5' and 3' FRT sites flanking the neomycin cassette on all 8 ES clones was verified. For Southern blots, 15 μg of gDNA was digested overnight with HindIII, run overnight on 0.8% agarose gels and transferred by capillarity to a Hybond-N+ nylon membrane (GE Healthcare). Prehybridization for 2 hours with ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion) was followed by hybridization overnight using a radioactively labeled probe for detection of the endogenous Tardbp gene.

Mouse Breeding

All protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Toronto Faculty of Medicine and Pharmacy Local Animal Care Committee as well as the University of Toronto Animal Care Committee. ES clones with the correct recombination event were used to obtain germline-transmitting chimeras by morula aggregation at the Toronto Centre for Phenogenomics (http://www.phenogenomics.ca/). Deletion of the neomycin selection cassette was achieved by breeding the F1 Tardbp targeted mice with Actin-FLP mice (Jackson laboratory, #003800). Excision of the neomycin cassette was verified using PCR flanking primers.

Quantitative Real Time PCR

Mice at 5 months of age were euthanized in a CO2 chamber and tissues were dissected and immediately snap-frozen in liquid nitrogen. Total RNA extraction was performed using RNeasy kit (Qiagen); 2 μg of total RNA was used to synthesize cDNAs with oligo(dT) and Superscript III Reverse Transcriptase (Life Technol-
Immunoblots

Mouse tissues (brain, spinal cord, liver) were dissected, snap-frozen and homogenized in SDS/urea buffer (0.5% SDS, 6M urea in phosphate buffered saline) for TDP-43 immunodetection or RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 1 mM EDTA, 0.1% SDS, protease inhibitor cocktail) and homogenized. Homogenates were analyzed on 10% SDS-polyacrylamide gels and transferred to PVDF membranes which were then blocked for 1 hour at room temperature with 5% skim milk powder in Tris-buffered saline (TBS). The membranes were then incubated overnight at 4°C with primary antibodies diluted in blocking solution as follows: mouse anti-mannos-binding lectin (Mbl) monoclonal antibody (MAB 3808) at 1:5000 dilution; anti-TDP-43 antibody (10782-2-AP, Proteintech) diluted 1:2000; or anti-Actin clone C4 (MAB 1501) diluted 1:5000. Immunoblots were then washed with TBS containing 0.05% Tween 20 and incubated for 1 hour at room temperature with anti-mouse or anti-rabbit IgG conjugated to peroxidase diluted 1:5000 in blocking solution. Antibody binding was detected with Western Lightning Plus ECL detection system (Perkin Elmer).

Results

To generate a conditional knockout of Tardbp we used the classical loxP-Cre recombination system. Using Bac recombinating technology, two loxP sites were introduced flanking exons 2 and 3 (Figure 1A). Deletion of exons 2 and 3 prevents translation from both the first translation start site in exon 2 and an alternate translation start site located in exon 3.

Homologous recombination between the targeting vector and the Tardbp locus in ES cells generated the Tardbp targeted allele. The targeting event was verified in ES clones surviving neomycin selection by Southern blot and sequencing (Figure 1B). ES clone 3G was empirically selected to generate germ-line transmitting chimeras which were crossed to C57BL/6 mice. In order to remove the flipped neomycin selection cassette, F1 Tardbp targeted mice were crossed to Actin-FLP mice and neomycin excision was confirmed by PCR amplification using primers flanking the FRT sites (Figure 1C). Heterozygous mice bearing floxed exons 2 and 3 at the Tardbp locus were designated as Tardbp/loxPlox.

Genotyping of 71 live-born F2 mice from a cross between Tardbp/loxPlox heterozygotes showed 22 Tardbp/+, 49 Tardbp/loxPlox, and no Tardbp/loxPlox/loxPlox mice. The absence of Tardbp/loxPlox/loxPlox offspring was highly significant (p<0.0001, Chi-square test); the ratio of Tardbp/loxPlox/loxPlox to Tardbp/loxPlox mice (22:49) fit the expected 1:2 ratio, consistent with a purely recessive inheritance lethality in the homozygote. PCR genotyping of embryos at E12.5 and E18.5 days revealed no homozygotes, suggesting that lethality occurred prior to these time points, but was not further investigated.

Since it has previously been shown that homozygous Tardbp knockout mice are embryonic lethal [11–13], it is possible that the inability to obtain homozygous Tardbp/loxPlox/loxPlox mice was due to an inadvertent effect of the gene targeting event on the expression of Tardbp. Analysis of Tardbp mRNA transcripts in lumbar spinal cord revealed no diminishment in TDP-43 expression between Tardbp/loxPlox mice and wildtype littermates and this was verified at the protein level by immunoblot analysis of brain, spinal cord and liver tissue (Figure 2A, 2B). However, since TDP-43 can auto-regulate its own expression levels the possibility that the targeting event disrupted Tardbp expression cannot be excluded [14,15].

An alternate explanation for the absence of Tardbp/loxPlox/loxPlox homozygote mice could be an indirect effect occurring as a consequence of the homologous recombination event at the Tardbp locus. Even though the homology arms of the targeting vector were specifically designed not to overlap with adjacent gene sequences, it is possible that the homologous recombination event affected the expression of neighboring genes.

In the mouse genome, the 3‘ untranslated region (UTR) of the Tardbp gene overlaps with the 2 last exons of the Masp2 gene. The Masp2 gene encodes for two isoforms, MAP19 is the short isoform (Figure 2C). These results show that targeting of the Tardbp locus affects expression of the adjacent gene, Masp2.

Discussion

In this investigation we generated heterozygous mice for a conditional knockout of the Tardbp gene by flanking exons 2 and 3 with loxP recombination sites. We found that while heterozygote Tardbp/loxPlox mice were viable and fertile, we were unable to obtain homozygotes. Since Tardbp knockout mice are embryonic lethal [11–13], we checked whether there was a loss of TDP-43 expression from the targeted allele. There was no apparent reduction in TDP-43 expression at either the mRNA or protein level, which might be real or a result of auto-regulation of the Tardbp wildtype allele [14,15], as has been reported in other Tardbp homozygote knockout mice [11–13]. We also considered whether the targeting event disrupted adjacent genes. In silico design of the targeting vector is one of the most important steps in generating gene knockouts in mice. Since homologous recombination of a
targeting vector is a rare event [16], our targeting vector was designed with long homology arms to increase the frequency of targeted integration at the *Tardbp* locus [17], while avoiding overlap with genes located upstream and downstream of *Tardbp*.

Figure 2. Analysis of *Tardbp* and Masp2 expression in *Tardbp*<sup>2lox+</sup> mice. A) Quantification of mRNA in mouse lumbar spinal cord by Real-Time PCR. No significant difference was found between *Tardbp* transcripts between WT and *Tardbp*<sup>+2lox+</sup> mice (n = 4). B) Immunoblot analysis of TDP-43 protein expression in mouse brain, spinal cord, and liver shows no reduction in expression in *Tardbp*<sup>+2lox+</sup> mice compared to wildtype littermates. Actin was used to normalize the amount of protein loaded. C) Quantification of *Masp2* splicing variants mRNA in mouse liver by quantitative Real-Time PCR. *Masp2* long variant was significantly reduced in *Tardbp*<sup>+2lox+</sup> compared to WT (p = 0.03, n = 3). No significant difference was found for *MAp19* transcript levels between WT and *Tardbp*<sup>+2lox+</sup> mice. D) Analysis of total protein extracts from mouse liver by Western blot. *Masp2* is clearly reduced in *Tardbp*<sup>+2lox+</sup> mice livers when compared to WT, while *MAp19* expression is marginally reduced.

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The 5′ homology arm of the targeting vector (Chr4: 148.625.922–148.631.862) was 5.9 Kb long and the 3′ homology arm (Chr4: 148.617.735–148.621.728) 3.9 Kb; the latter is located 2.23 Kb downstream of the 3′ UTR of *Masp2*, and as such would not be
expected to affect expression of this gene. Nevertheless, we found a significant reduction in both mRNA and protein expression of the long isoform of Masp2 in heterozygote Tardbp2lox/lox mice compared to their non-transgenic littermates, indicating that the targeting did affect Masp2 and that this could underlie the inability to obtain homozygous Tardbp2lox mice. Of note, the greater effect on Masp2 compared to Map19 might relate to the overlap of the last 2 exons of Masp2 with the 3’UTR of Tardbp.

The reduced expression of Masp2 could be the result of an introduction of unexpected mutations in the chromosome by the recombination event at the Tardbp locus. Even though homologous recombination generally occurs with high fidelity [18], many investigations have reported the incorporation of mutations upon targeted homologous recombination [19–21]. Moreover, the fidelity of homologous recombination can be compromised when the sequences of the targeting vector and the chromosome are not isogenic [18]. In our study, the RP23-29102 BAC used to make the targeting vector had a C57BL/6J background while the mouse Embryonic Stem cells were from a hybrid 129/C57BL6 background. It is possible that the homologous recombination event at Tardbp introduced mutations that altered regulatory sequences governing the expression of the Masp2 gene. Even though regulatory sequences have been identified upstream of the Masp2 gene [22,23], the regulation of this gene remains poorly understood. Some of the Masp2 regulatory enhancers could reside downstream of the gene, as has been reported for other genes [24,25].

Masp2 deficiency in mice confers protection from gastrointestinal ischemia/reperfusion injury and are viable in a homozygous state [26], which would seem contradictory to our inability to obtain homozygous Tardbp2lox+/lox Masp2 deficient mice. However, this discrepancy could be due to the mouse strain background of the previously reported Masp2 deficient mouse, which was established in 129/Sv embryonic stem cells, whereas Tardbp2lox+/lox mice were in a hybrid C57BL/129 strain background. It is well recognized that the strain background has a relevant influence in phenotype of transgenic mice [27,28].

There are relatively few publiciations on the generation and use of Tardbp conditional knockout mice for the study of ALS [29–31]. Chinag et al., 2010 floxed Tardbp exon 3 and found that ubiquitous deletion of Tardbp leads to a metabolic phenotype in embryonic stem cells. Wu et al., 2012 showed that deletion of Tardbp exons 2 and 3 in heterozygote mice leads to an age dependent progressive motor dysfunction. However, to date neither group has reported on homozygote mice. Iuchi et al., 2013 obtained homozygote mice with a conditional deletion of Tardbp exon 2, after backcrossing for at least 5 generations, and showed an age dependent motor dysfunction. Thus, generation of TDP-43 deficient mice has been a challenging task and in this investigation we show for the first time an effect of targeting Tardbp on downstream gene Masp2.

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Author Contributions

Conceived and designed the experiments: SD DM. Analyzed the data: SD. Wrote the paper: SD JR.

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