Therapeutic rAAVrh.10-mediated SOD1 silencing in adult SOD1^{G93A} mice and non-human primates.

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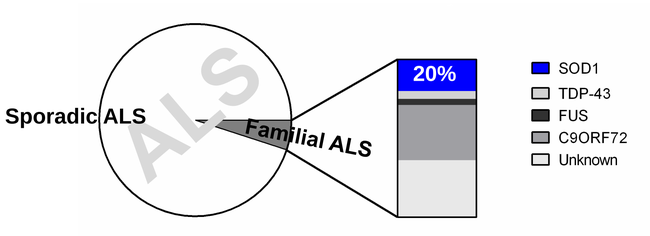
SOD1-linked ALS

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease; survival in ALS is typically 3-5 years. No treatment extends patient survival by more than 2-3 months. Mutations in Cu/Zn cytosolic superoxide dismutase (SOD1) have been associated with 20% of fALS.



The disease arises from a dominant gain-of-function. Silencing of both wild-type and mutated SOD1 is therefore a potential therapeutic strategy. In fact, a 60% reduction in SOD1 protein was shown to slow disease progression after onset in a mouse model of the disease (Foust *et al.,* 2013).

Study design

An artificial microRNA termed miR-SOD1 targets a region of the SOD1 mRNA that is conserved in most mutants and across primates. In these studies, it is expressed either from a polymerase II (chicken β-actin) or pol III promoter (U6), and is delivered *in vivo* by a recombinant adeno-associated vector serotype rh10 (rAAV.rh10) to a mouse model of SOD1-linked ALS as well as non-human primates.

<u>Hypothesis</u>: 1) rAAV.rh10-mediated SOD1 silencing will be therapeutic and prolong survival in adult-treated SOD1^{G93A} mice.

2) Lumbosacral intrathecal injection of rAAV.rh10-miR-SOD1 in marmosets will mediate SOD1 silencing in motor neurons along the entire spinal cord.

In vitro validation of silencing candidate

The artificial miRNA miR-SOD1 (Fig.1a) was designed to target human and primate SOD1 (Fig.1d). Transfection of miR-SOD1 in HEK293 cells lead to SOD1 silencing at mRNA level (Fig.1b) and protein reduction (Fig.1c).

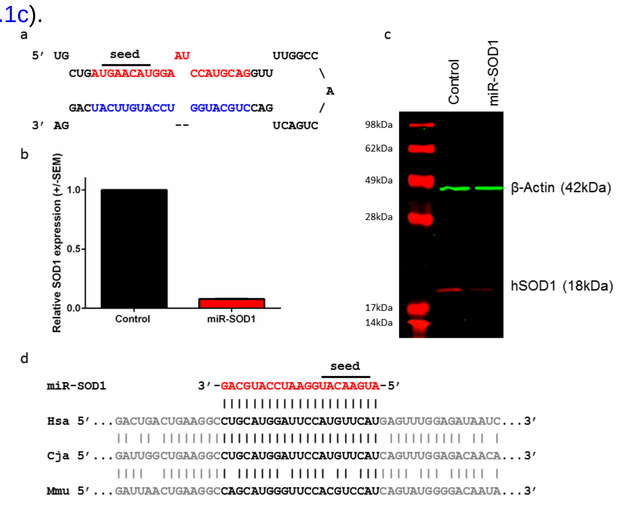


Figure 1. Design and *in vitro* validation of miR-SOD1.

SOD1 silencing is therapeutic in adult mice

The transgenic SOD1^{G93A} mouse is an aggressive model of ALS, due to a high copy number of the human SOD1^{G93A} transgene. The mice exhibit a decline in hind limb strength and motor function, and over time become paralyzed. Priori to endpoint, rapid weight loss is caused by decreased tongue force, and breathing rapidly deteriorates, leading to death.
Treatment of adult mice, while highly relevant from a clinical perspective, has proven challenging. Here, adult mice (55-68 days) were treated systemically with a dose of 2E11 genome copies of vector expressing miR-SOD1. Control and treated groups were litter-matched to account for the high variability of this model. The mice were euthanized upon blinded assessment by an experienced animal caretaker.

Survival was extended by 20% with the CB-miR-SOD1 treatment (Fig.2a) and by 29% with the U6-miR-SOD1 treatment (Fig.2b). Disease onset is delayed by 12.5% with the CB-miR-SOD1 treatment (Fig.2c) and by 31% with the U6-miR-SOD1 (Fig.2d) treatment. Disease duration was prolonged by 94% with the CB-miR-SOD1 treatment (Fig.2e) and was not affected by the U6-miR-SOD1 treatment (Fig.2f).

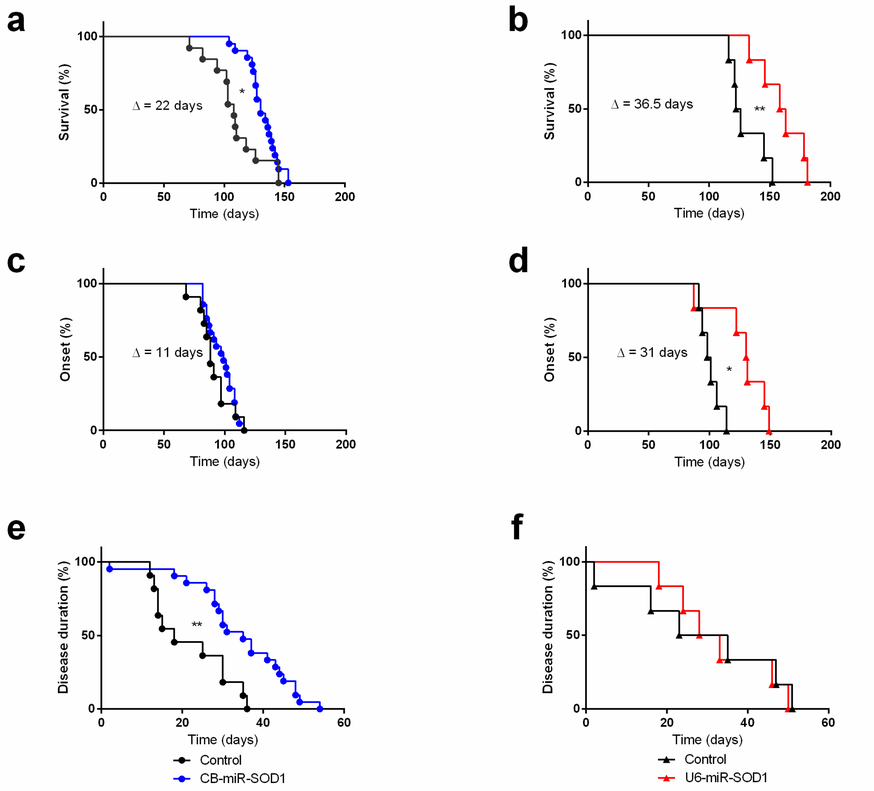


Figure 2. Reducing SOD1 expression prolongs survival in SOD1^{G93A} mice.

General motor skills and limb strength of the animals were assessed (Fig.3) by quantification of the latency to fall during a rotarod performance test (Fig.3a), and 2 (Fig.3b) or 4 limbs grip strength (Fig.3c).

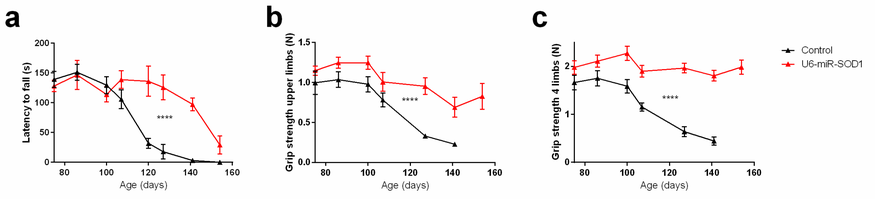


Figure 3. Reducing SOD1 expression preserves motor skills and limb strength.

Marmoset motor neurons are transduced

Marmoset monkeys received 6E12 genome copies per kg body weight of vector expressing miR-SOD1intrathecally and were euthanized 20 days post-injection.

NHP motor neurons are transduced by rAAVrh.10. Fixed spinal cord tissue was sectioned at 40µm and incubated with an anti-GFP antibody. Representative images are presented here, with Fig.4d-f being a higher magnification of Fig.4a-c showing specifically the motor neuron-rich ventral horns. (Fig.4g-i) Frozen spinal cord tissue was sectioned at 20µm and motor neurons were laser-captured by an experienced technician. Both motor neurons (Fig.4i) and non-motor neurons tissue (Fig.4h) were harvested.

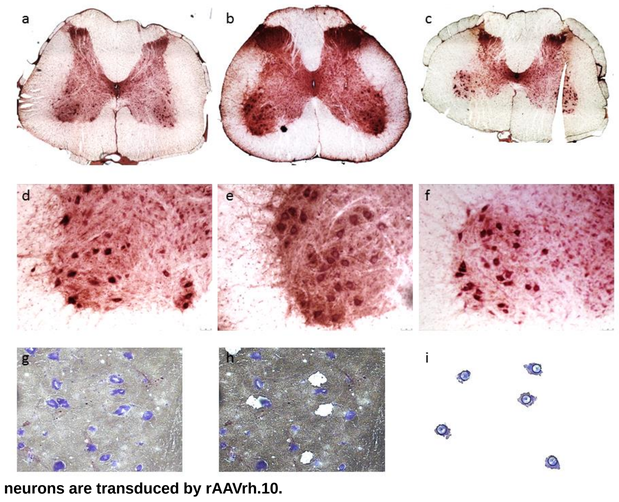


Figure 4. NHP motor neurons are transduced by rAAVrh.10.

SOD1 silencing in non-human primates

Next, SOD1 and Hprt transcripts were quantified by RT-qPCR in spinal cord motor neuron (Fig.5a-c), non-motor neuron (Fig.5d-f), and pons/medulla (Fig.5g) tissue. Mature miR-SOD1 was quantified by RT-qPCR in spinal cord motor neurons (Fig.5h). Spinal cords were sectioned into lumbar (Fig.5a, d, h), thoracic (Fig.5b, e) and cervical (Fig.5c, f).

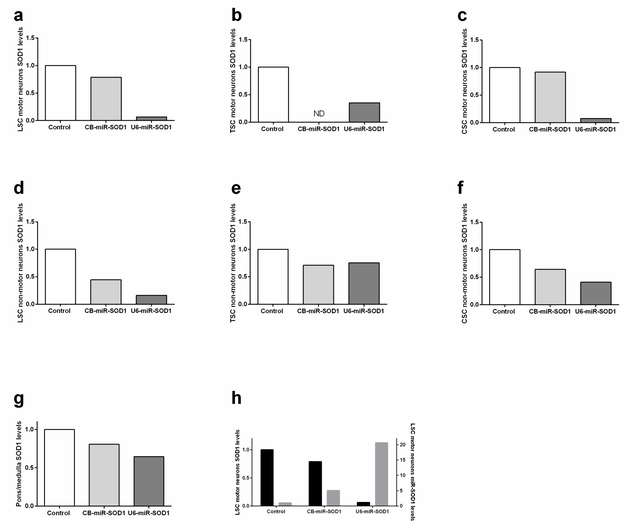


Figure 5. SOD1 silencing in NHP motor neurons and pons/medulla, correlates positively with the levels of mature miR-SOD1.

Finally, 3-plex RNA *in situ* hybridization (ISH) was done on 20µm frozen spinal cord sections (Fig.6), with probes designed to recognize GFP (in green), SOD1 (in yellow) and mature cholinergic neuron (including motor neuron) marker choline acetyltransferase (ChAT, in red). Nuclei were counterstained with DAPI (in blue). Spinal cords are from the control-treated animal (Fig.6a-b) or the U6-miR-SOD1-treated animal (Fig.6c-d).

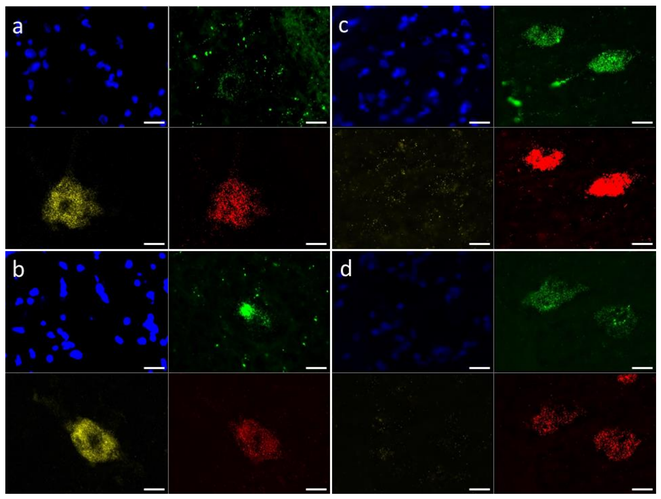


Figure 6. SOD1 mRNA is undetectable in GFP-positive, ChAT-positive motor neurons of animal treated with U6-miR-SOD1.

Conclusion

In conclusion, we show that silencing SOD1 with rAAVrh.10-miR-SOD1 is therapeutic in adult SOD1^{G93A} mice, which establishes a solid proof-of-concept. To our knowledge, this is the longest extension achieved to date by gene therapy initiated in adult ALS mice.

In non-human primates, we demonstrate robust SOD1 knockdown. In addition these results document efficient vector spread from the injection site all the way to the brain stem and the brain, hence demonstrating that the intrathecal route would be a suitable delivery method in a clinical trial.

Future work will investigate improvements to the delivery protocol to ensure reproducibility and further assessment of promoters.

Acknowledgements

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