FGFBP1 gene therapy promotes functional improvement in skeletal muscle of SOD1^{G93A} ALS mice.

"U-stem Master: Stem cells and genome editing"
2019/2020

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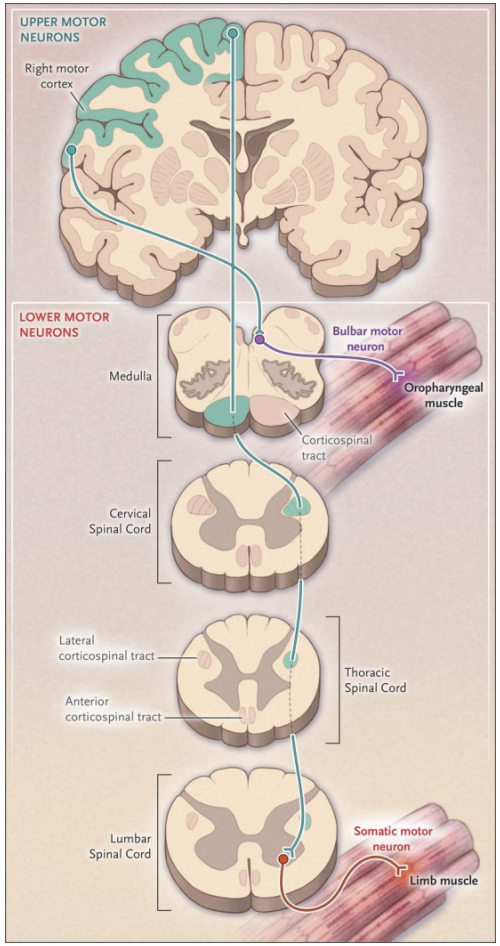
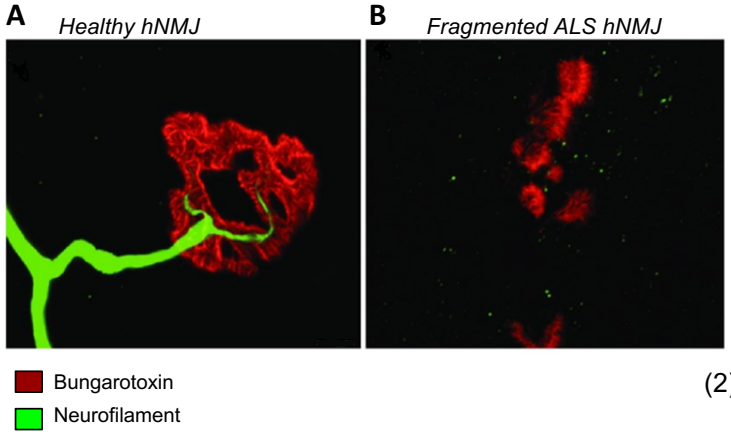
Background:



(1)

The disease:

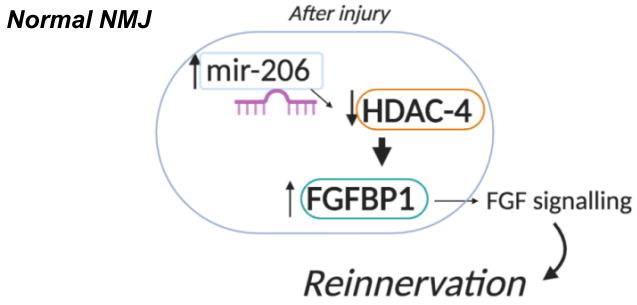
- Amyotrophic lateral sclerosis (**ALS**)
- Multifactorial and multigenic, no cure available;
- Death ~ 3-5 years after diagnosis: respiratory failure;
- Early symptoms: muscle atrophy and dismantlement of neuromuscular junction (**NMJ**);

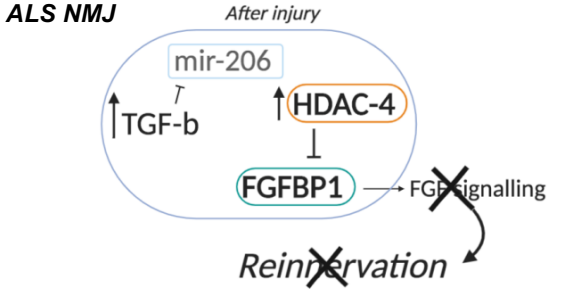


(2)

Degeneration of NMJs:

- The stability of the NMJ requires instructive molecular signals (muscle>neuron);
- FGF signaling is involved in NMJ reinnervation and his maintenance.





(3)

Fig. 1) Adapted from ref1); Fig. 2) Adapted from ref 2; Fig 3) Originally produced.

AIM of the project: gene therapy upregulating FGFBP1

Why FGFBP1?

- Previous studies of FGFBP1 in SOD1^{G93A} mice (fig 4-5)
- It does not interfere with other pathways in skeletal muscle

Why AAV-2 vector?

- Low immunogenicity
- Previous studies in skeletal muscle of SOD1^{G93A} mice
- Delivery System: AAV-2 from Vector Biolabs

Cell line: C2C12

- Rapid maturation into functional skeletal muscle cells
- Robust model to check viability and proliferation after transfection

Animal model: SOD1^{G93A}

- Most used animal model in ALS studies
- Phenotype reflects the one of human disease

Cell line: myoblasts from SOD1^{G93A} mouse + iPSCs-derived motor neurons

- Model of an ALS affected NMJ on-a-chip

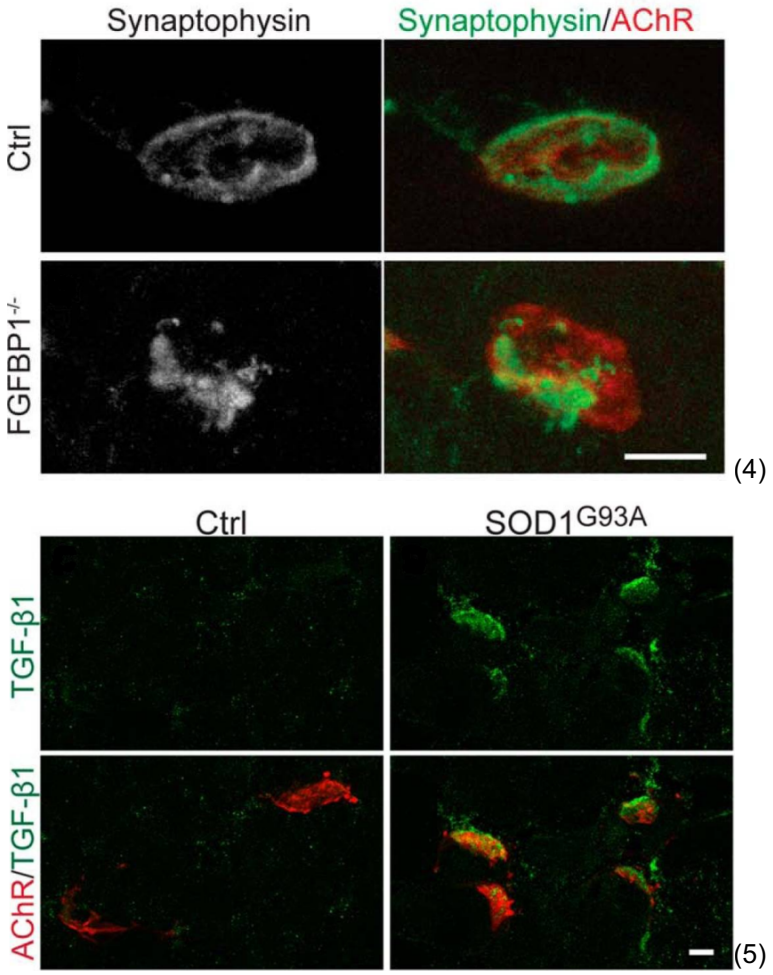


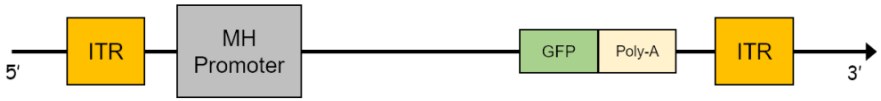
Fig. 4) and 5): adapted from ref.3

In vitro expression of the transgene:

Genome copies per transfection: $1*10^{10}$

AAV2 FGFBP1 vector:

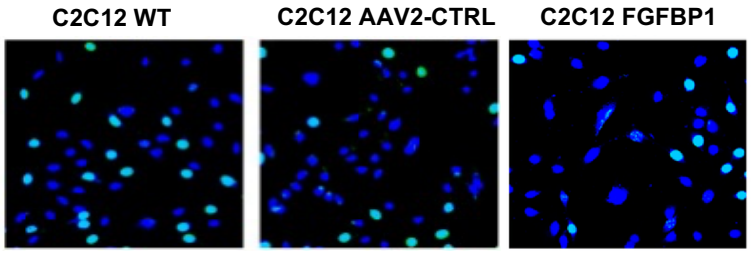
AAV2-CTRL vector:

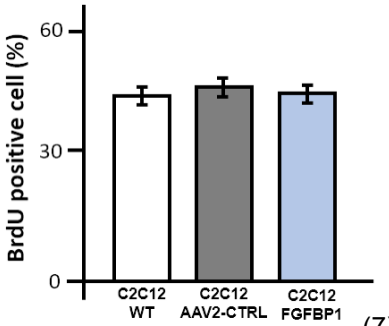


(6)

How do C2C12 respond to the vector?

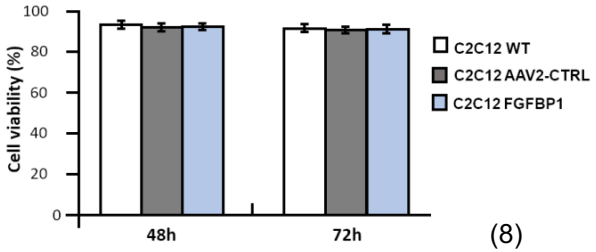
Proliferation test





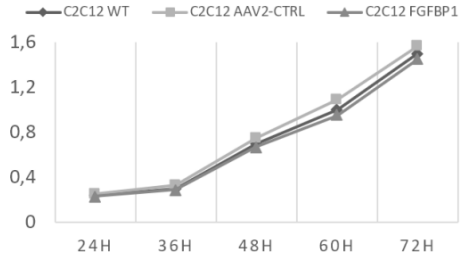
(7)

Viability test



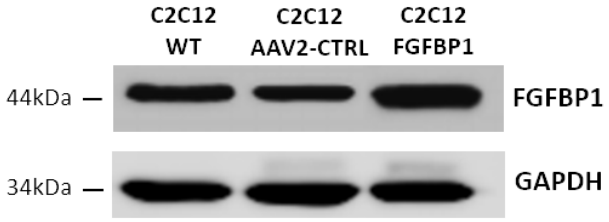
(8)

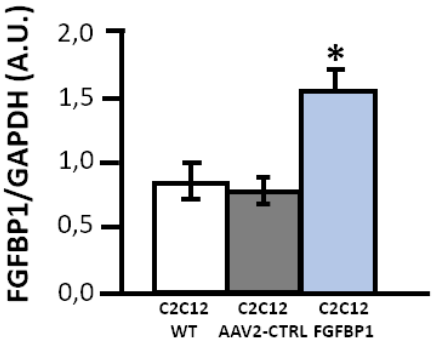
MTT Assay



(9)

Have we got differences in the expression of FGFBP1?





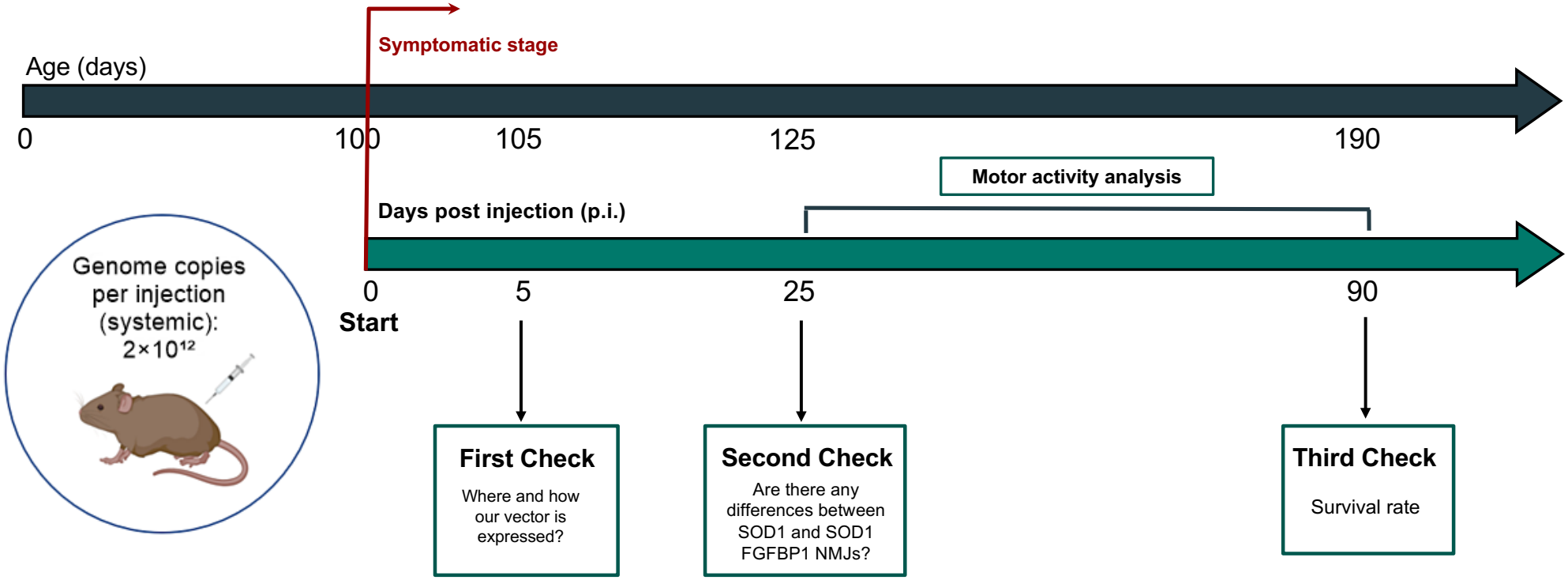
(10)

Fig. 6) Originally produced; Fig. 7) and 8) Adapted from ref4; Fig 9) originally produced; fig. 10) Adapted from ref5.

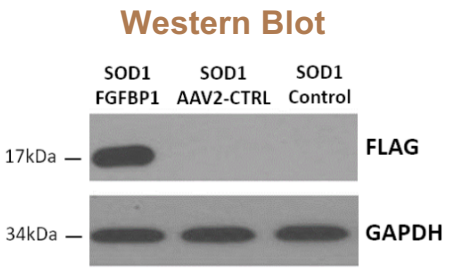
In vivo expression of the transgene:

At least 3 mice per group for every "check":

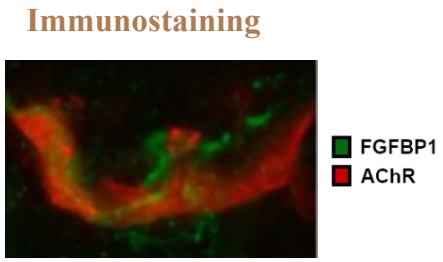
1. **SOD1 Control**: $SOD1^{G93A}$ Untreated
2. **SOD1 AAV2-CTRL**: $SOD1^{G93A}$ injected with AAV2 not expressing FGFBP1 sequence
3. **SOD1 FGFBP1**: $SOD1^{G93A}$ injected with AAV2 expressing FGFBP1 sequence
4. **WT AAV-CTRL**: Wild type injected with AAV2 not expressing FGFBP1 sequence
5. **WT FGFBP1**: Wild Type injected with AAV2 expressing FGFBP1 sequence
6. **WT Control**: Wild Type



Where is our vector expressed?



(11)



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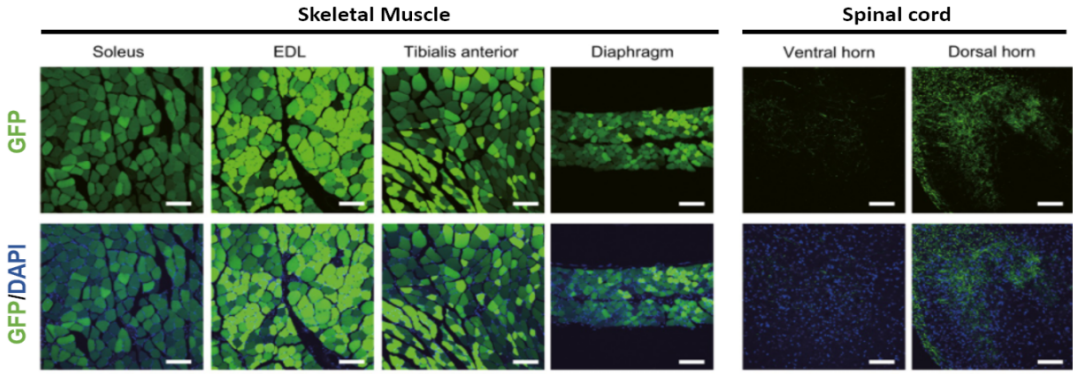
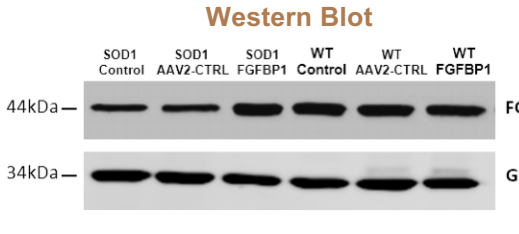
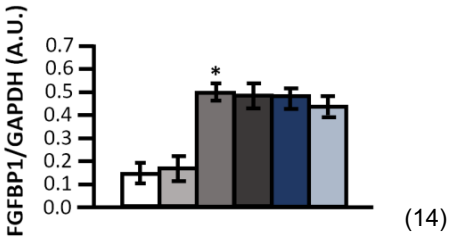
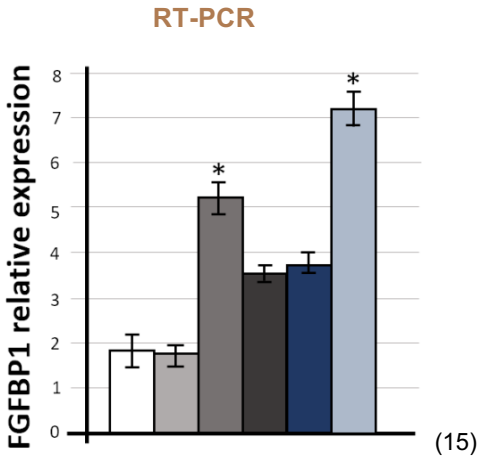


How much is our vector expressed?





(14)



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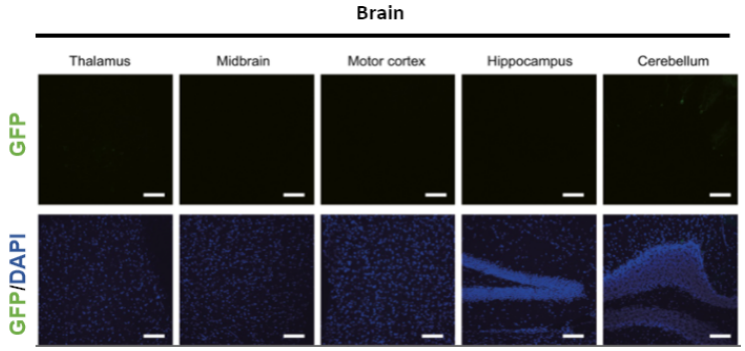
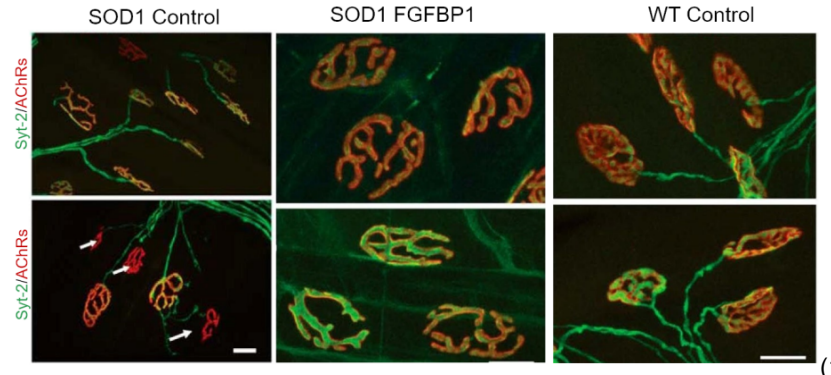


(13)

Fig. 11) originally produced; Fig. 12) Adapted from ref3; Fig. 13):adapted from ref.8 . Fig. 14) Adapted from ref.5; Fig15) Originally produced

Are there any differences between SOD1 and SOD1 FGFBP1 NMJs?



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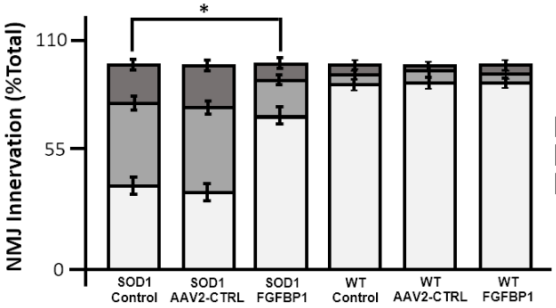
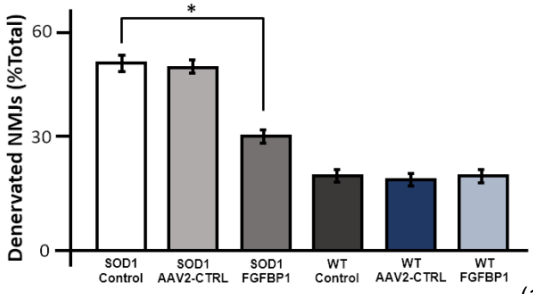
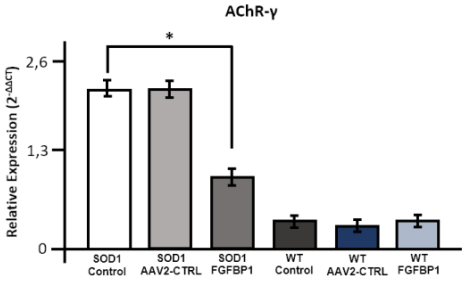
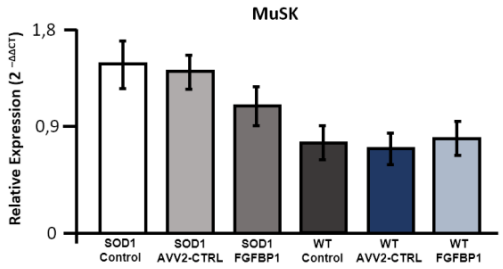
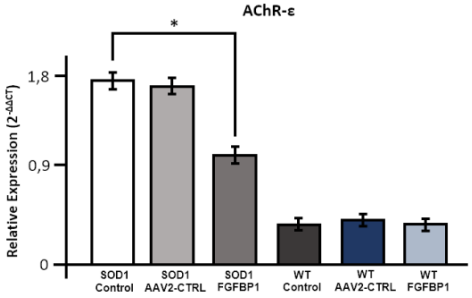


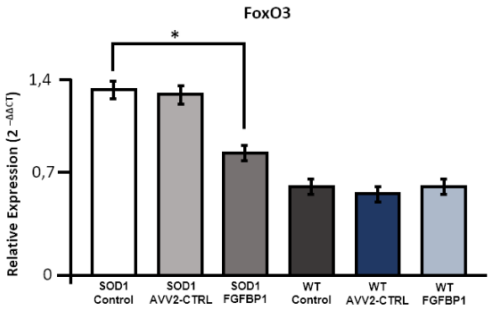


(17)









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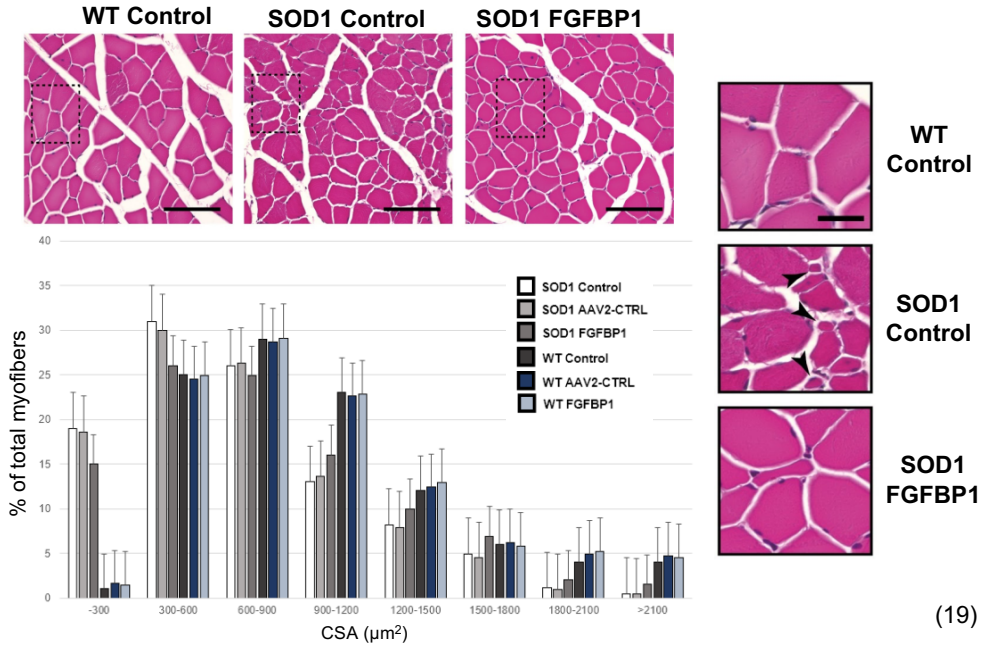
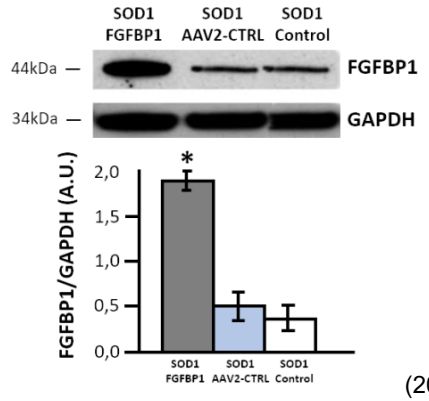


(19)

Fig. 16-18) Adapted from ref.3; fig.19) from ref.8

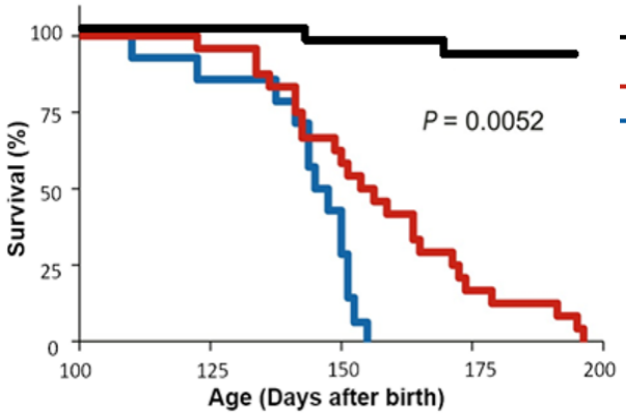
Survival rate and Motor activity

Western Blot 50 days p.i.

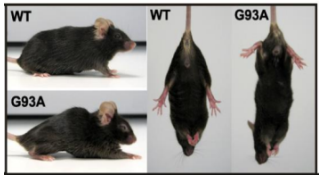


(20)

Kaplan–Meier survival curves

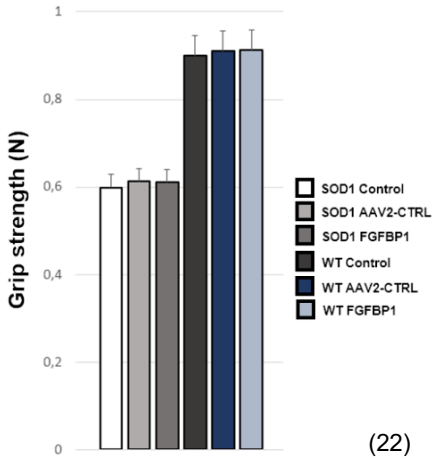


(21a)



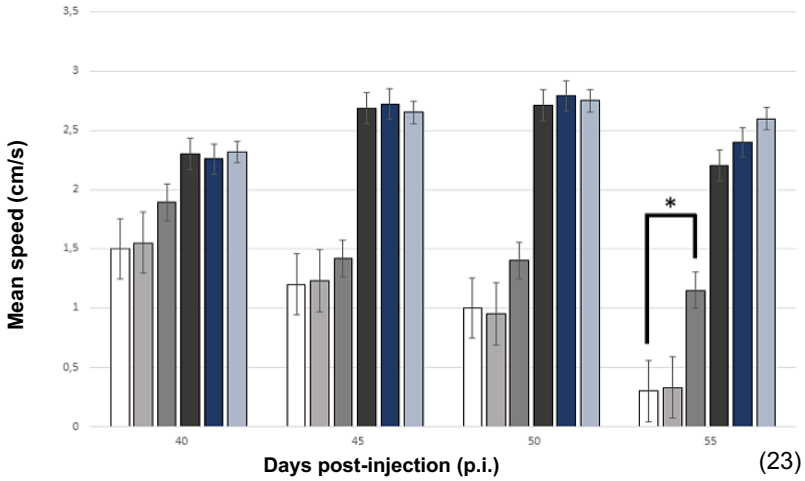
(21b)

Grip test



(22)

Open field test



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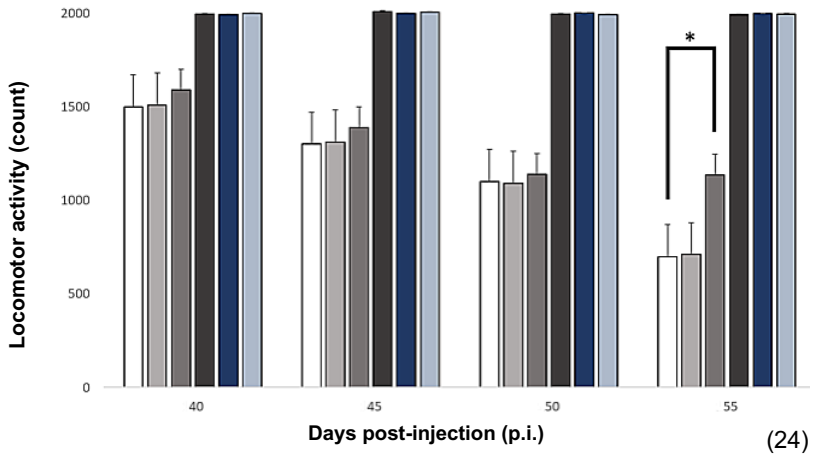
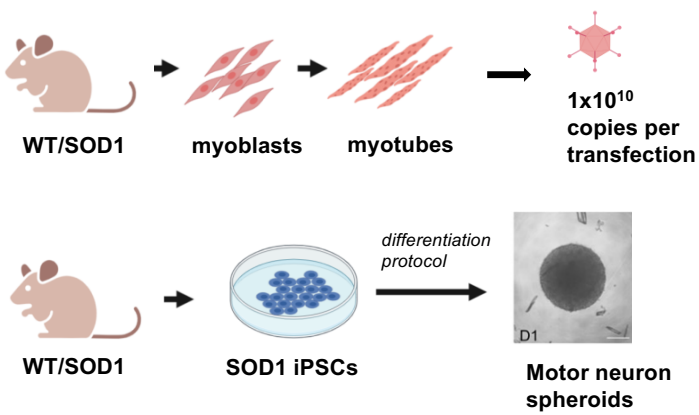


(24)

Fig. 20) from ref 9; fig. 21a), 22)-24) from ref.8;

Supplementary experiment: NMJ-on-a-chip



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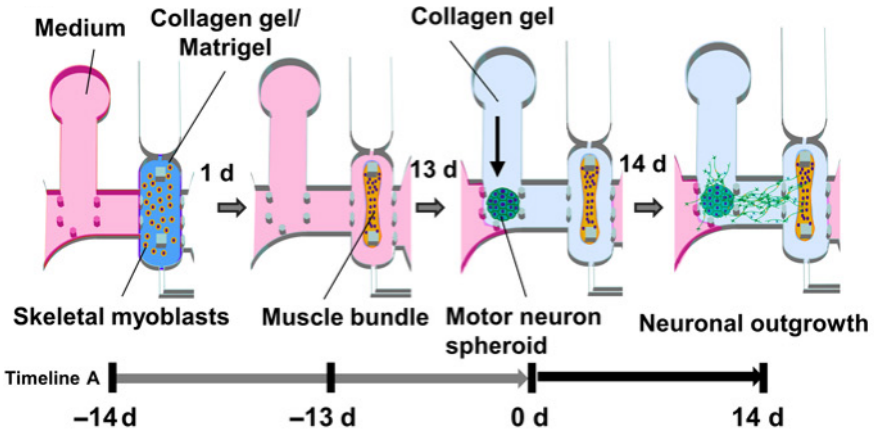
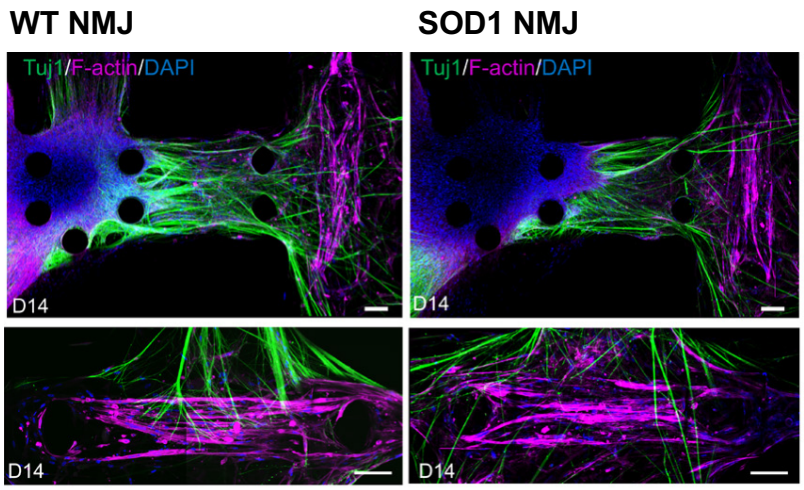


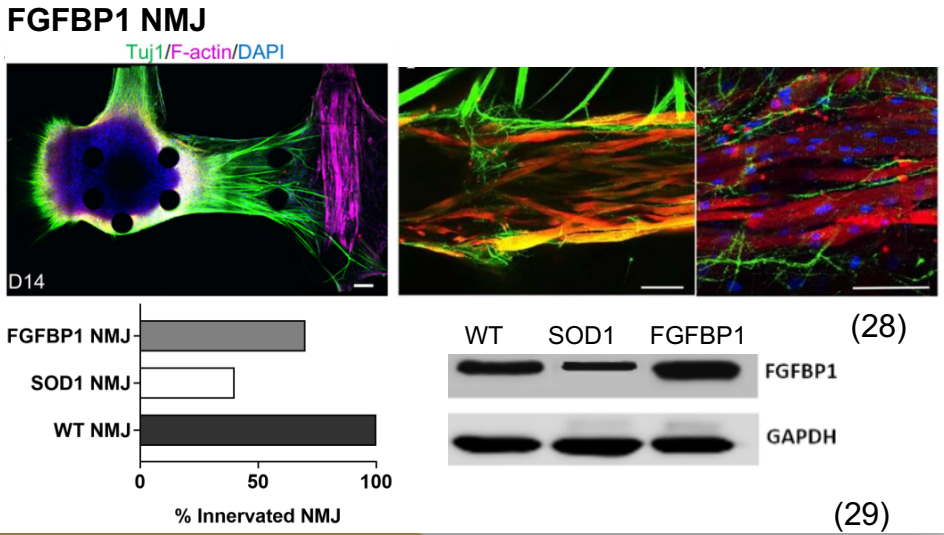
(26)



(27)

+AAV2-FGFBP1

Fewer thick neural fibers and less NMJ formation



(28)

(29)

Fig. 25) Originally produced+photo from ref.6; Fig. 26), 27) and photo on the left in fig.28): from ref.6. Photos on the right in 28) from ref.7; fig.29): WB from ref. 5, histogram originally produced

Conclusions:

- **Robust** and **specific** expression of the transgene (exogenous **FGFBP1**) in muscle cells: no off-target effects.
- SOD1 mice up-regulating FGFBP1 show **larger and healthier muscular fibers** than SOD1 mice, similarly to wild type animals.
- Up-regulation of FGFBP1 at muscular level results in **less fragmented NMJs**, increasing the rate of **innervation**.
- SOD1 mice expressing the AAV-2 FGFBP1 vector show **improved motility** and a **higher survival rate** than untreated mice.

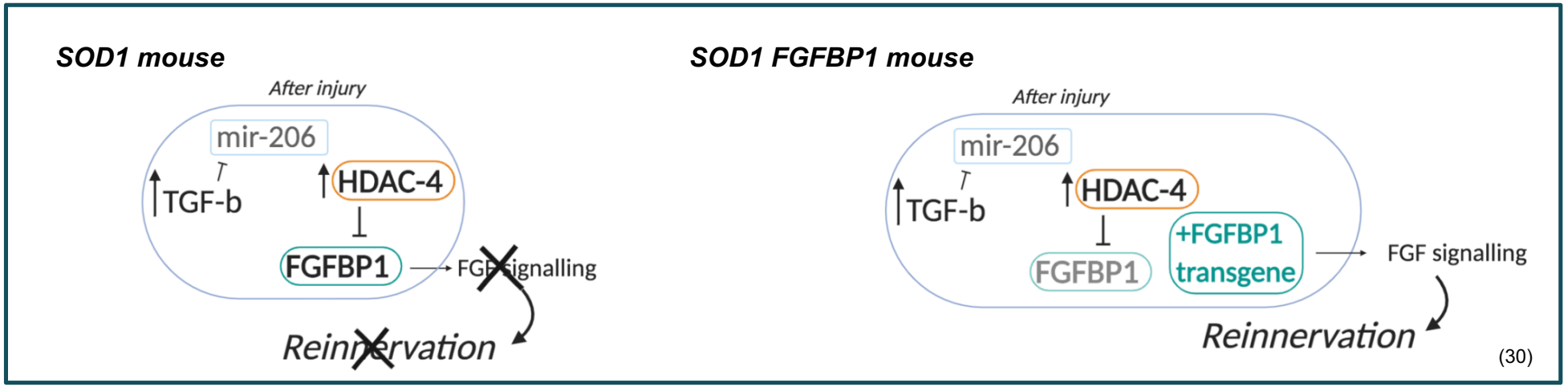


Fig. 30) originally produced.

Pitfalls and Solution

AAV-2 FGFBP1 silenced

The expression of the transgene could decrease with time, even if the promoter is strong.

Multiple injections

If the expression of FGFBP1 would decrease during lifetime, multiple injections of the viral vector should be performed.

Difficulty in evaluating the *in vivo* motor activity

Grip test/open field test are highly influenced by the temporary attitude of the animal, so they have limited reproducibility.

Repeating the tests on a larger sample

Using more mice should give a more precise idea of the partially recovered muscular strength of the animals.

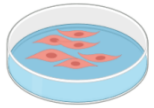
Materials and Methods:

AAV-2 vector bought from Vector BioLabs, with FGFBP1 transgene (+flag) tagged with GFP and expressed under the control of MH promoter (muscle hybrid, highly specific for muscle cells. See ref.11). Transfect *in vitro* on undifferentiated C2C12 mice muscle cells (ThermoFisher®) in triplicate, using two control groups of cells (C2C12 non-transfected and C2C12 transfected with a GFP-only vector).
Supplementary: Isolate myoblasts from SOD1 and WT mice through muscle extraction, transfect with viral vector and culture on chip. Isolate mouse embryonic fibroblasts (MEFs) and de-differentiate into iPSCs; then differentiate motor neuron spheroids iPSCs-derived and co-culture on chip together with muscular cells.

Systemic injections of the vector ($2.5x10^{12}$ vg AAV2 in 30 µL PBS) in 10 mice per group (5 males and 5 females), for a total of 60 mice (6 groups). Start the treatment at an age of 100 days (symptomatic stage). Sacrifice three mice per groups at different times for protein analysis and histological analysis. Finally, use three mice per group for evaluation of survival rate.

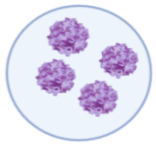
RT-PCR and Western Blot (WB) to evaluate the intensity of expression and the localization of AAV-2 FGFBP1 expression (in biological triplicate for each group). **Histological analysis (*in vivo* section)** of muscular fibers and NMJs 25 days post-injection through haematoxylin-eosin and immunofluorescence stainings, respectively for macroscopic (muscular fibers dimension) and microscopic (NMJs integrity) evaluation. To do in biological triplicate for each group. **Motor activity analysis (grip and open field test)** from day 25 p.i. onwards, repeat on the highest possible number of animals (visible results from 45 days p.i.). **Histological analysis of NMJ-on-a-chip** at the confocal microscope, WB to confirm the presence of FGFBP1 and count of innervated NMJs**.**

Costs: starting from 87.000€



C2C12+ Lab equipment:
about 3.500/4.000€

Viability/ proliferation/MTT assays:
about 800€

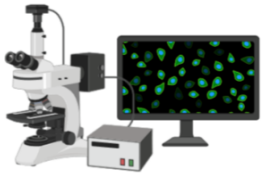


AAV-2 vector:
about 19.000€

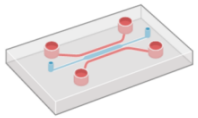
Western Blot, RT PCR analysis:
about 2.000€

Motor activity analysis (grip test):
about 2.000€

Mice (SOD1G93A+ Wild Type):
about 10.000€ (excluding maintenance)



Visual analysis:
about 2.000€



Microfluidic device + iPSCs equipment:
about 7.000€

One PhD student:
About 40.000 € (3/4 years salary)

References

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2. Bruneteau G, Simonet T, Bauché S, et al. Muscle histone deacetylase 4 upregulation in amyotrophic lateral sclerosis: potential role in reinnervation ability and disease progression. *Brain*. 2013;136(Pt 8):2359-2368. doi:10.1093/brain/awt164
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