17 DECEMBER 2019

A THERAPEUTIC STRATEGY TO BLOCK PROGERIN PRODUCTION IN HUTCHINSON-GILFORD PROGERIA SYNDROME, VIA AAV9 EXPRESSION VECTORS

by Clara Arqué, Mattia Fumagalli and Carlos González

Background

THE DISEASE

- Hutchinson-Gildford Progeria Syndrome (HGPS)
- De novo autosomal dominant disease
- Premature aging, postnatal growth retardation, early loss of body weight,
- Death ~13 years old: heart attack or stroke
- Loss VSMC --> fibrosis --> atherosclerosis

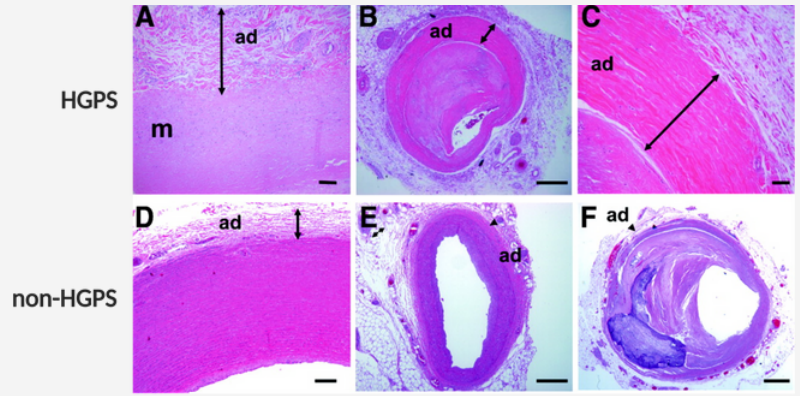


Fig. 1 Fibrosis of the adventitia in HGPS
Immunohistological analysis of cardiovascular tissues from two children with HGPS (A-B-C) and
comparative analyses with nonHGPS cohorts (D-E-F)

THE GENE: LMNA

- Encodes for Lamin A/C
- Intermediate filaments of nuclear lamina
- Control nuclear shape, DNA replication and gene expression

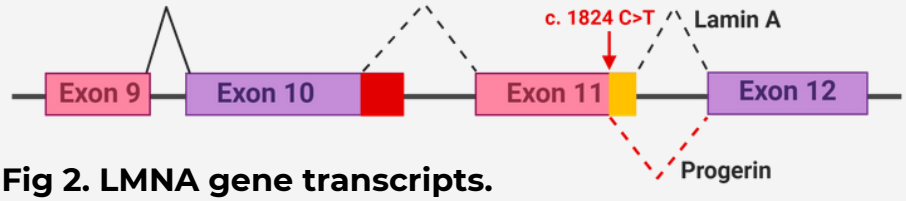
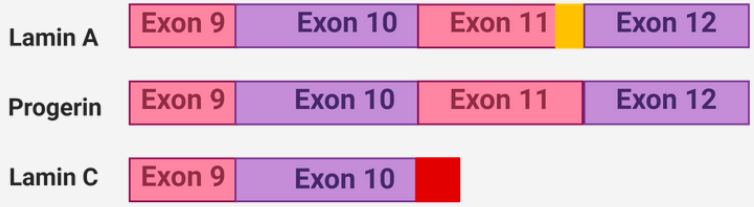


Fig 2. LMNA gene transcripts.
Specific part of exon 10 for lamin C (in red).
150 nucleotide region of exon 11 deleted in progerin (in yellow)

THE MUTATION: C.1824 C>T

- Activation of a cryptic splicing site => deletion of 50 aa
- Abnormal processing
- Farnesylated lamin A isoform => progerin



Aim of the project

shRNAs progerin knockdown

WHY shRNA?

- Knockdown of target gene
- Low rate of degradation
- Previous studies with shRNAs and HGPS

WHY AAV9 VECTOR?

- Low immunogenicity
- Infection of dividing and non-dividing cells
- Episomal concatemers, no insertional mutagenesis
- Serotype 9 infects more affected tissues (heart, muscle)

CELL LINE: HGPS FIBROBLASTS

- One of the more affected cells in HGPS
- Easy to manipulate and work with

ANIMAL MODEL: TRANSGENIC HGPS MICE

- Contains the human LMNA gene carrying the p.G608G mutation
- Phenotype similar to humans
- We studied the most affected tissues: heart and muscle

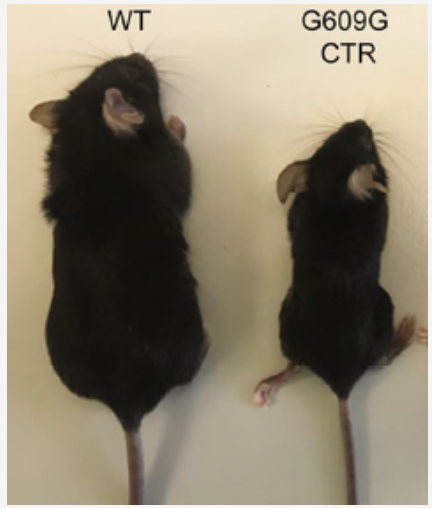


Fig 3. WT and G609G CTR female mice at 3.5 months of age.

Vector design

DELIVERY SYSTEM

AAV9 vectors from Vector Biolabs

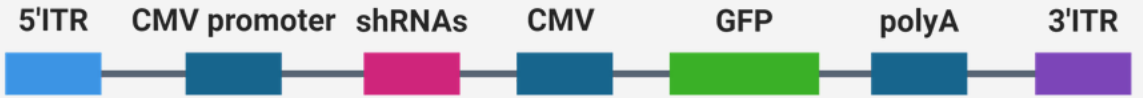


Figure 3: Structure of AAV9 vector.

shRNA SEQUENCES

- Size: ~2.2kb
- 4 different shRNAs + 1 scrambled shRNA
- BLAST to check the similarity of the sequences

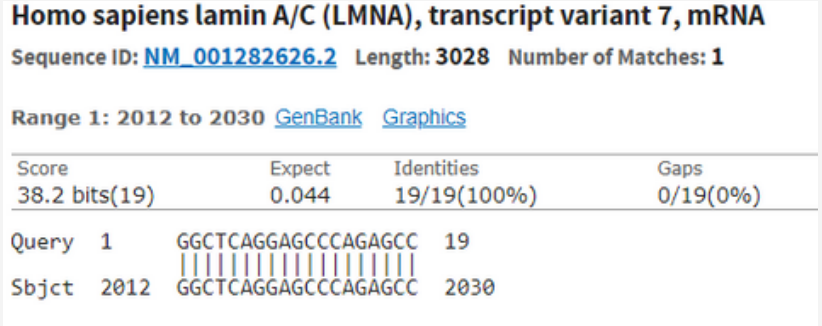


Figure 4:BLAST of shRNA1 vs progerin mRNA

shRNA1 (from Huang et at):
5'- *GGCT*CAGGAGCCCAGAGCC*CCTT-CAAGA*GAGGGGCTCTGGGCTCCTGAG*CC* -3'
shRNA2 (designed with siDirect 2.0 software):
5'- *ACAT*GATGCTGCAGTTCTG*GGTT-CAACC*CAGAACTGCAGCATCAT*GT* -3'
shRNA3 (designed with BLOCK-iT™ RNAi Designer software):
5'- *CACC*GCGGGCAGCCTGCCGACAAGG*GGTT-CAACC*CCTTGTCGGCAGGCTGCCC*GC* -3'
shRNA4 (designed with siDirect 2.0 software):
5'-*CACC*G*TTTTTCTTTGGCTTCAAGCCC*GGTT-CAACC*GGGCTTGAAGCCAAAGAAA*AA*-3'
Scrambled shRNA (designed with siRNA Wizard software):
5'- *ACCTC*GCGCACCGCAGGGAGACCT*TCAA-GAGA*GGTCTCCCTGCGGTGCGC*TT* -3'

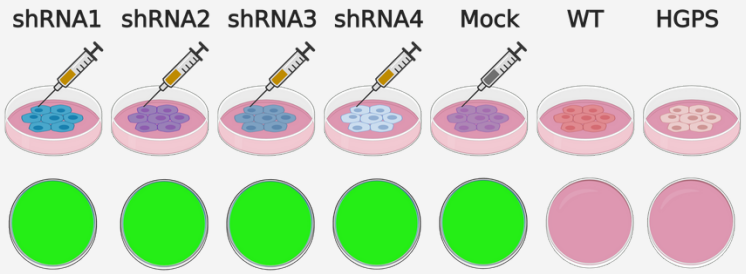
Figure 5: Sequences of the shRNAs. Sequences in italics are the linkers and the loops

In vitro therapy

Genome copies per injection:
1*10^10

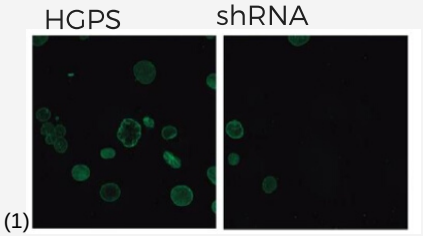
1) FLUORIMETRIC MEASURE OF INFECTION RATES

2) STUDIES ON INFECTED CELL CULTURES

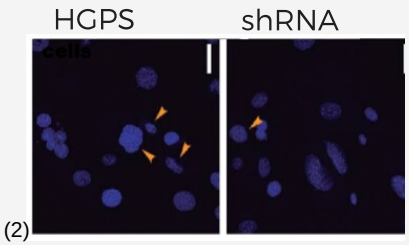


3) CELL SORTING OF TRANSFECTED CELLS

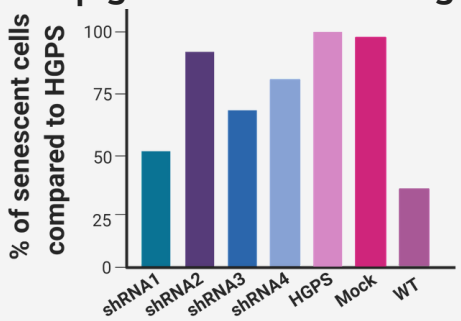
Progerin immunostaining



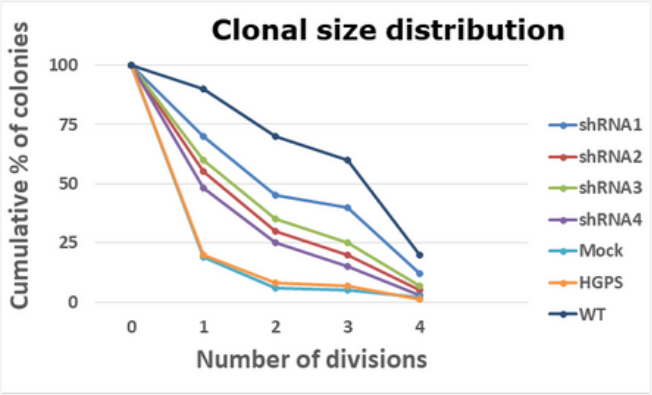
DAPI colouration



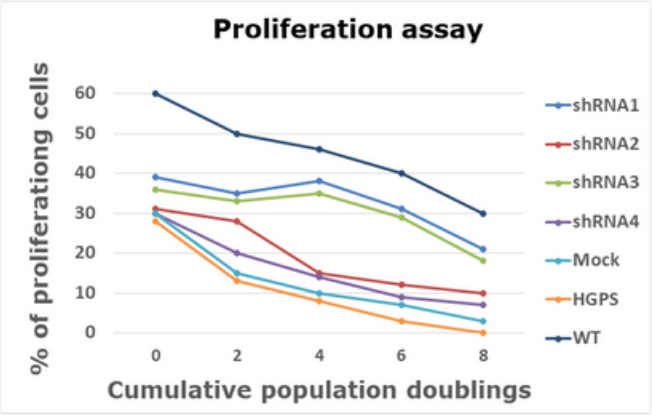
β-galactosidase staining



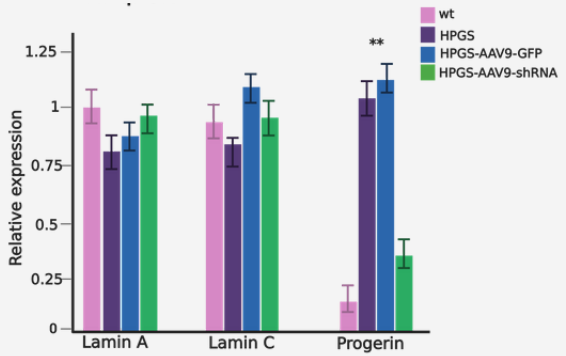
Clonal size distribution



Proliferation assay



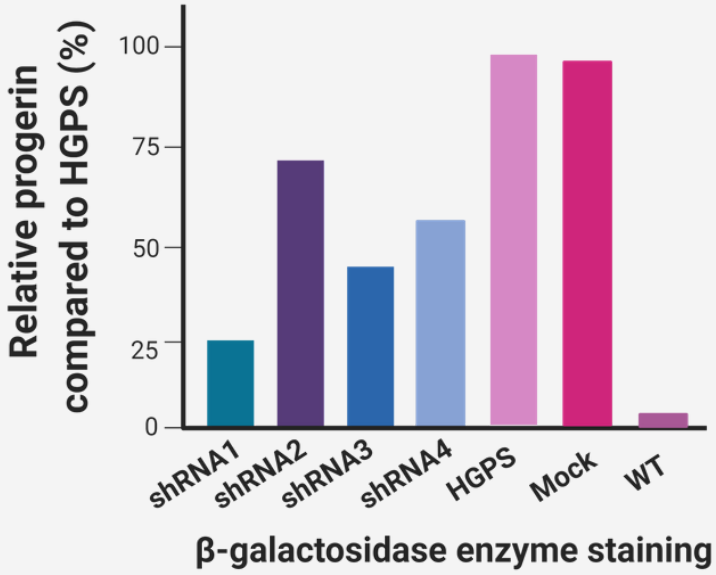
qPCRs for mRNAs



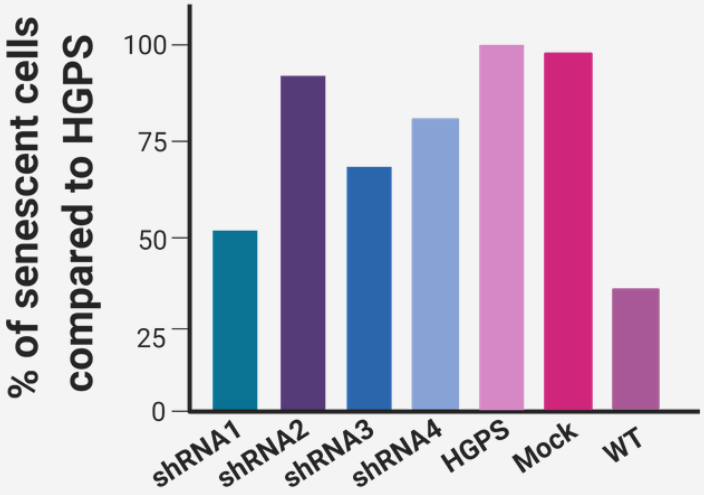
Western Blot

fig 1,2,3 are adapted from ref. 3 and 4, other images are originally produced

Choice of the shRNA vector and safety control



- We choose the shRNA which present a better yield in the previous tests
- The chosen vector is transferred to 5 wt colonies
- these colonies are used to measure proliferation capacity and senescence ratios, to clarify possible deleterious side effects



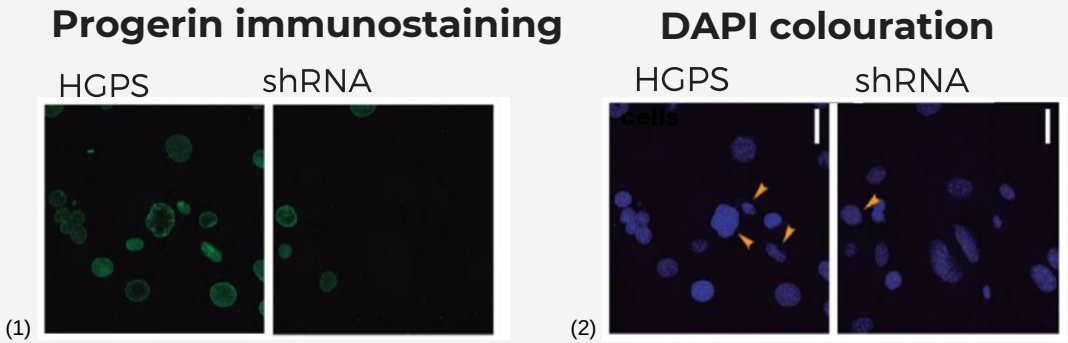


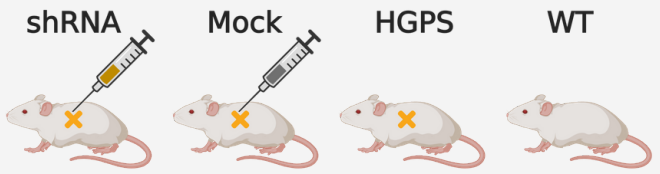
fig 1,2 are adapted from ref. 4, other images are originally produced

In vivo therapy

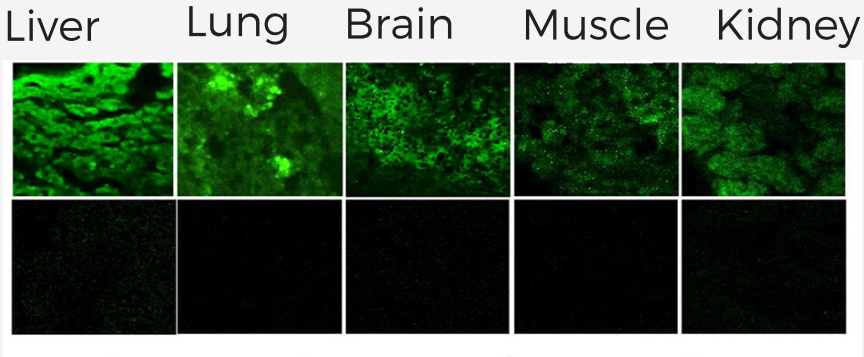
Genome copies per injection:
2*10^12
Solution: 40 µl PBS + virus

MICE MODELS INFECTION

- Mice are transfected with the best shRNA or the mock version.
- The therapy is administrated by intraperitoneal injection.
- 4 mice for each group are sacrified 48h after the injection,
- We measure the infection in several organs, with more interest in those ones most affected by HGPS.



STUDY OF THE INFECTION BY TISSUE FLUORESCENCE



(1)

CELL ISOLATION FOR CYTOFLUORIMETRIC STUDY

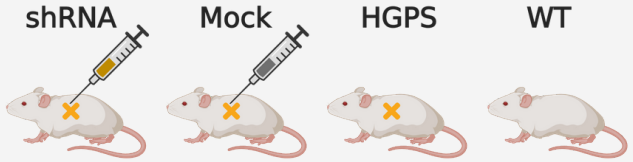
INFECTION RATES MEASUREMENT

fig 1 is adapted from https://doi.org/10.3389/fphar.2017.00241, other images are originally produced

In vivo therapy

MOLECULAR, CELLULAR AND HISTOLOGICAL ASPECTS

Genome copies per injection: 2*10^12
Solution: 40 µl PBS + virus



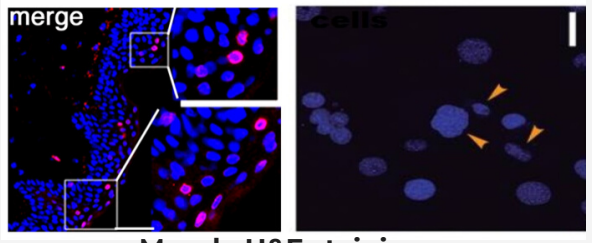
1) CELL SORTING OF TRANSFECTED CELLS IN DIFFERENT TISSUES → WESTERN BLOT AND qPCR

2) HISTOLOGICAL ANALYSIS OF HGPS SYMPTOMS IN DIFFERENT TISSUES

Fluorescence measurement

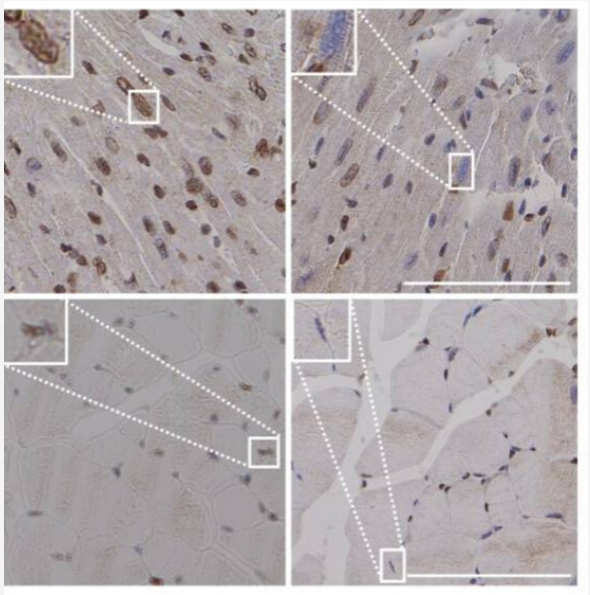
(1)

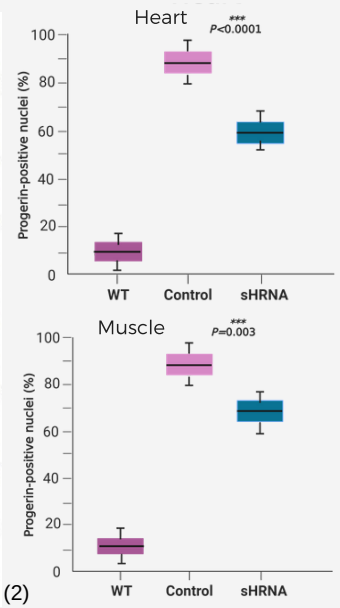
DAPI nuclear shape evaluation



(3)

Progerin & Lamin A immunostaining





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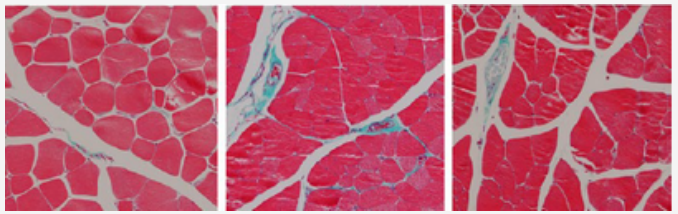
Muscle H&E staining



(4)

Immunogenicity test

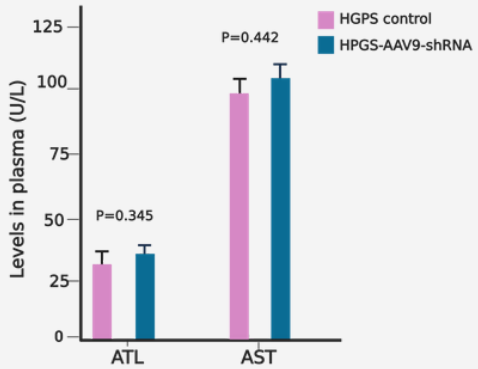


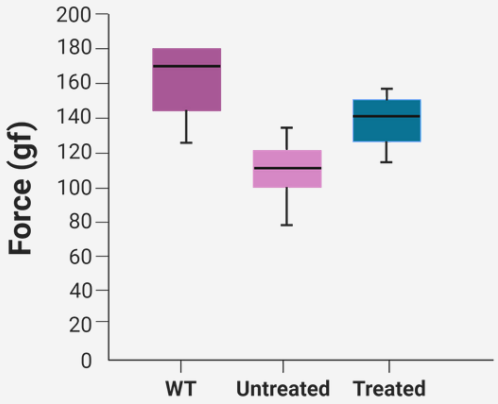
fig 1,2,3,4 are adapted from ref. 3 and 4, other images are originally produced

In vivo therapy

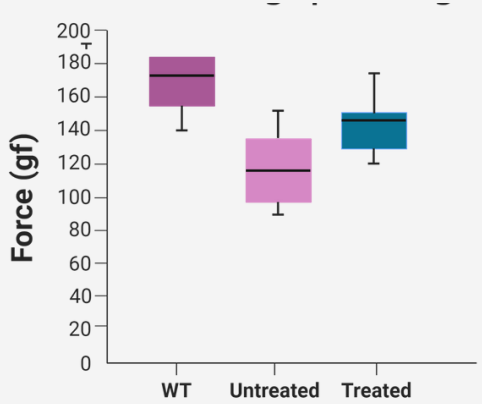
HEALTH AND LIFESPAN MEASUREMENT

10 mice from each different group are monitored for a set of phenotypic traits indicative of the severity of progeria. They are let live until natural death, to measure their lifespan

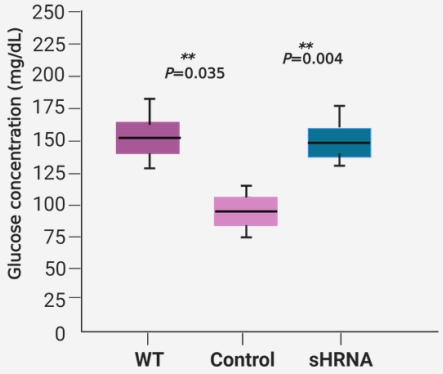
Forelimb grip strenght



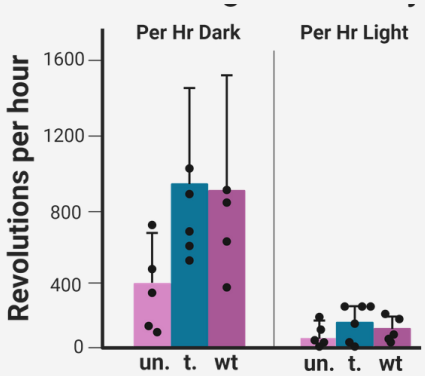
Hindlimb grip strenght



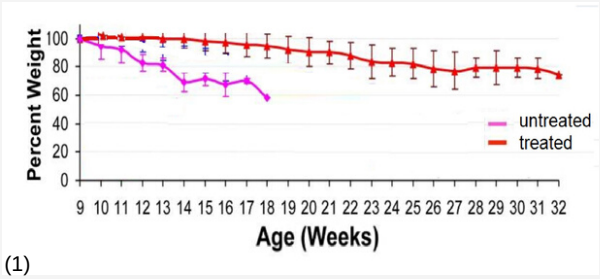
Blood glucose levels



Running wheel assay

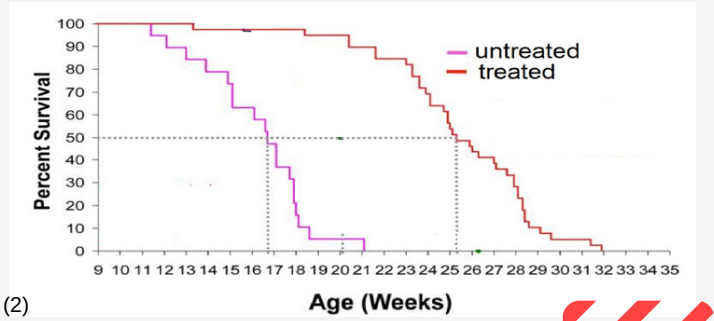


Body weight progression



(1)

Survival



(2)

fig 1,2 are adapted from ref. 4, other images are originally produced

MATERIALS AND METHODS

VECTORS AND MODELS

- Mice models: wild type C57BL/6 mice and transgenic mice for human progeria mutated gene C57BL/6-Tg(LMNA*G608G)HClns/J
- Cell cultures: untransformed human fibroblasts from progeria patients
- Viral vectors: AAV9 vectors, with EGFP expression cassette, expressing either a shRNA among the 4 chosen or a scrambled RNA.

METHODS FOR IN VITRO STUDIES

Infection rates are measured with a cell sorter in 3 colonies per group. Then, 3 colonies of treated, untreated HGPS and Wild-type fibroblast undergo: clonal size and proliferation assay; beta galactosidase staining assay; immunostaining for progerin; studies on nuclear shape by DAPI staining. In 4 colonies for every group infected cells sorted by the cytofluorimeter undergo Western blot for LMNA/LMNC/Progerin; qPCR on mRNAs for the same genes.

METHODS FOR IN VIVO STUDIES

Infection rates in tissues are measured by fluorescence and cell sorting, in 4 mice per group. 10 mice from each group are screened through time for weight, activity, limb strength and glucose levels then to compare lifespans. At 3,5 months of age, in other 6 mice: tissue morphology vectors expression, immunotoxicity and nuclear shape of transfected cells are observed. Infected cells are isolated and selected by cytofluorimetry for western blot and qPCR.

BUDGET

234.000€

without relatively generic laboratory equipment

37.429€

Mice lines

4.938€

Cell lines and products

9.895€

Virus production

210€

Viability tests

2.091€

Single cell suspensions

256€

Histological analysis

1.642€

Progerin immunofluorescence

2.422€

WT blot, RNA extraction and RT-qPCR

1.298€

Protein insolation and immunoblot analysis

12233€

Physiologic analysis

176.000€

Salaries per year for:
1 PI
2 PhD students
1 tecnhician

PITFALLS AND SOLUTIONS

Contrasting results

Tests for phenotypic parameters for shRNA therapies could give contrasting results for progerin expression and other symptoms.

Vector toxicity

The vector we apply may result to cause reduced viability or proliferation in the safety control.

Non-integrating vector

AAV9 is a non-integrating vector due to the removal of rep genes. There is a limited transmission to daughter cells

Tests' repetition

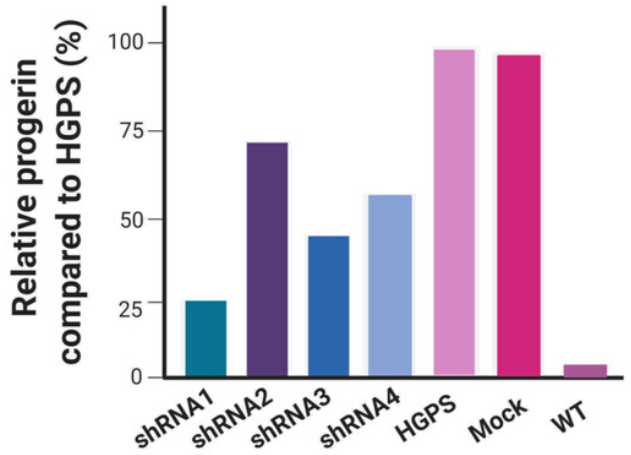
This kind of discrepancy is not expected for the known mechanism of the pathology, So if detected, the tests are to be repeated

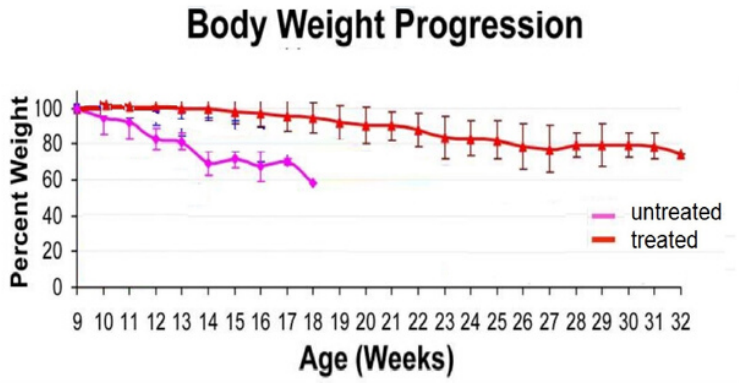
Choose the 2nd best

After repetition of the safety check, if the toxicity is confirmed, the 2nd best shRNA vector is tested instead.

Periodic treatment

In case of non-significant results, we could repeat the experiment by applying more than one injection throughout the development, to increase the number of transfected cells





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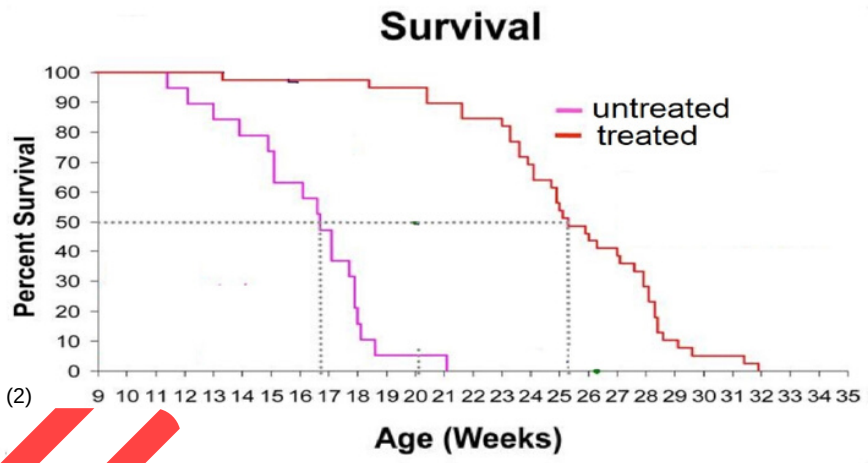

(2)

Conclusions

We expect significant and widespread knock -down of progerin, in mice tissues, low or no immunotoxicity of the vector and decisive phenotype amelioration. The shRNA "per se" is not expected to have severe side effects. Moreover AAV9 is reputed to be a very safe, non integrating transfection vector, which is reported to elicit no significant immune response. Therefore, also in the light of the extremely dramatic pathology profile, our results could already open the way to the translation to clinical trials.

(2)

fig 1,2 are adapted from ref. 4, the other image is originally produced

References

1. Piekarowicz K, Machowska M, Dzianisava V, Rzepecki R. Hutchinson-Gilford Progeria Syndrome—Current Status and Prospects for Gene Therapy Treatment. Cells. 2019;8(2):88. doi:10.3390/cells8020088
2. Huang, S., Chen, L., Libina, N. et al.Correction of cellular phenotypes of Hutchinson-Gilford Progeria cells by RNA interference Hum Genet 2005; 118: 444. https://doi.org/10.1007/s00439-005-0051-7
3. Beyret E, Liao HK, Yamamoto M, et al. Single-dose CRISPR-Cas9 therapy extends lifespan of mice with Hutchinson-Gilford progeria syndrome. Nat Med. 2019;25(3):419–422. https://doi.org/10.1038/s41591-019-0343-4
4. Santiago-Fernández, O., Osorio, F.G., Quesada, V. et al. Development of a CRISPR/Cas9-based therapy for Hutchinson–Gilford progeria syndrome. Nat Med 2019;25, 423–426 https://doi.org/10.1038/s41591-018-0338-6
5. Ellis, B.L., Hirsch, M.L., Barker, J.C. et al. A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with Nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype. Virol J 10, 74 (2013) https://doi.org/10.1186/1743-422X-10-74
6. Guan Y, Ma Y, Li Q, et al. CRISPR/Cas9-mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse. EMBO Mol Med. 2016;8(5):477–488. https://doi.org/10.15252/emmm.201506039
7. Rodriguez, S., Coppedè, F., Sagelius, H., & Eriksson, M. (2009). Increased expression of the Hutchinson-Gilford progeria syndrome truncated lamin A transcript during cell aging. European Journal of Human Genetics, 17(7), 928–937. https://doi.org/10.1038/ejhg.2008.270
8. Huang, S., Chen, L., Libina, N., Janes, J., Martin, G. M., Campisi, J., & Oshima, J. (2005). Correction of cellular phenotypes of Hutchinson-Gilford Progeria cells by RNA interference. Human Genetics, 118(3–4), 444–450. https://doi.org/10.1007/s00439-005-0051-7
9. Olive, M., Harten, I., Mitchell, R., Beers, J., Djabali, K., Erdos, M. R., … Gordon, L. B. (2011). NIH Public Access, 30(11), 2301–2309. https://doi.org/10.1161/ATVBAHA.110.209460.Cardiovascular
10. Liu GH, Suzuki K, Qu J, et al. Targeted gene correction of laminopathy-associated LMNA mutations in patient-specific iPSCs. Cell Stem Cell. 2011;8(6):688-694. doi:10.1016/j.stem.2011.04.019
11. Brunetti-Pierri N, Ng P. Helper-Dependent Adenoviral Vectors. In: Adenoviral Vectors for Gene Therapy: Second Edition. Elsevier Inc.; 2016:423-450. doi:10.1016/B978-0-12-800276-6.00017-6
12. Fong LG, Ng JK, Lammerding J, et al. Prelamin A and lamin A appear to be dispensable in the nuclear lamina. J Clin Invest. 2006;116(3):743-752. doi:10.1172/JCI27125
13. Rodriguez S, Coppedè F, Sagelius H, Eriksson M. Increased expression of the Hutchinson–Gilford progeria syndrome truncated lamin A transcript during cell aging. 2009. doi:10.1038/ejhg.2008.270
14. Olive M, Harten I, Mitchell R, et al. Cardiovascular Pathology in Hutchinson-Gilford Progeria: Correlation with the Vascular Pathology of Aging. Arter Thromb Vasc Biol. 2010;30(11):2301-2309. doi:10.1161/ATVBAHA
15. Barrow KM, Perez-Campo FM, Ward CM. Use of the cytomegalovirus promoter for transient and stable transgene expression in mouse embryonic stem cells. Methods Mol Biol. 2006;329:283-294. doi:10.1385/1-59745-037-5:283
16. Moore CB, Guthrie EH, Huang MTH, Taxman DJ. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. Methods Mol Biol. 2010;629:141-158. doi:10.1007/978-1-60761-657-3_10
17. Naito Y, Yoshimura J, Morishita S, Ui-Tei K. SiDirect 2.0: Updated software for designing functional siRNA with reduced seed-dependent off-target effect. BMC Bioinformatics. 2009;10. doi:10.1186/1471-2105-10-392