M.Sc. Genetics and Molecular Biology
Gene Therapy
Prof. Isabella Saggio
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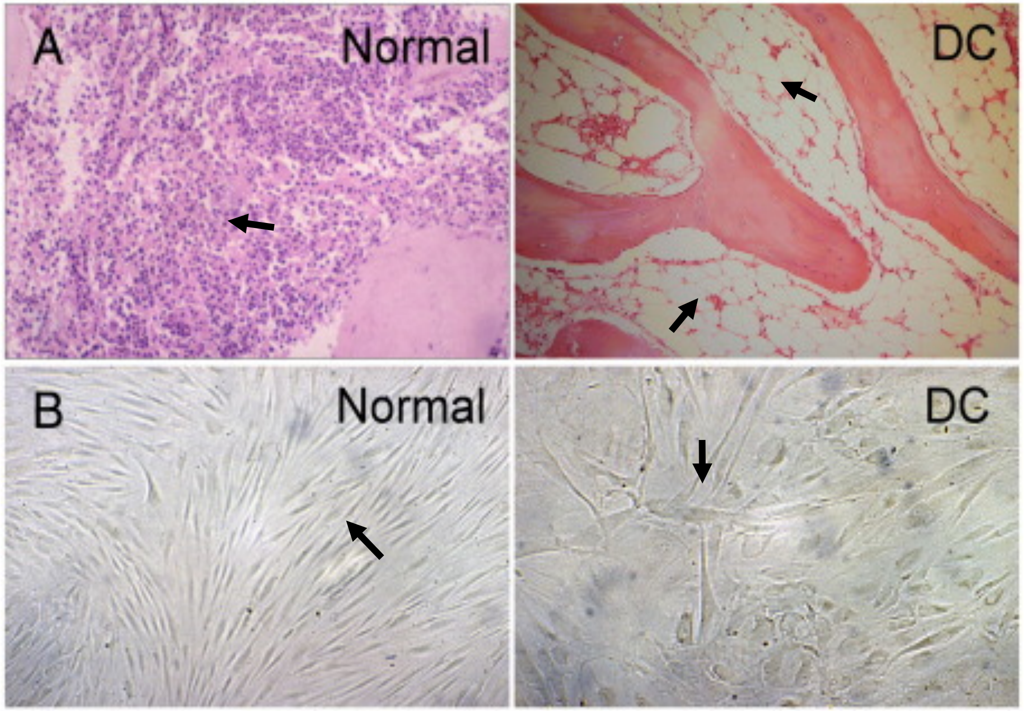
Gene Therapy for Dyskeratosis Congenita

Federica Farinella
Josune Imaz
Marta Laganà
Francesca Romana Liberati

Universitat Autònoma de Barcelona
Sapienza Università di Roma

What are the clinical features?

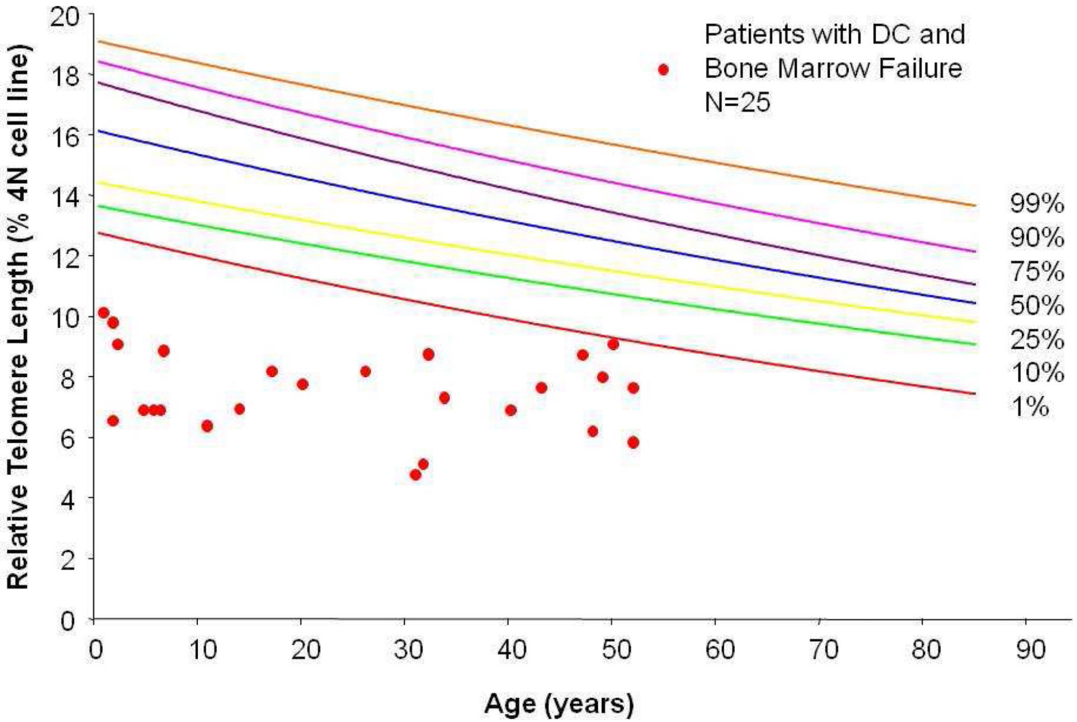


Kirwan,M. Dokal I, Biochim Biophys acta, 2009.

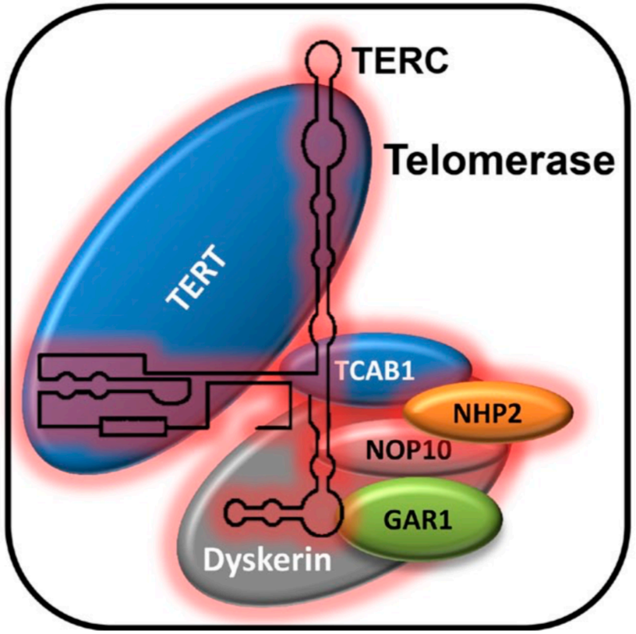
Incidence:
1 out of a Million

- Skin pigmentation
- Nail dystrophy
- Mucosal leukoplakia
- **Bone marrow failure**

What are the molecular features?



Masona P. J *et al.,* Cancer Genet. 2011.

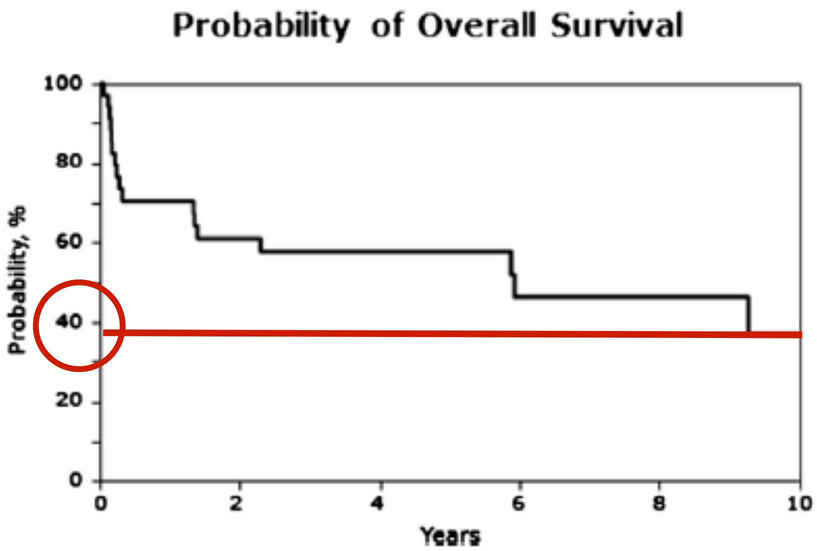


EXON 11 (A353V)

Holohan B. *et al.,* Cell biology of disease, 2014.

Objectives

Current therapy



Shahinaz M. Gadalla *et al.,* Biol Blood Marrow Transplant, 2013.

Our Goals

Main goal

Find a therapy that can substitute allogenic BM transplantation

Sub goals

- See how far we go manipulating HPSC
- Produce an accurate gene editing system

Experimental Plan

DKC1- Exon 11

↓

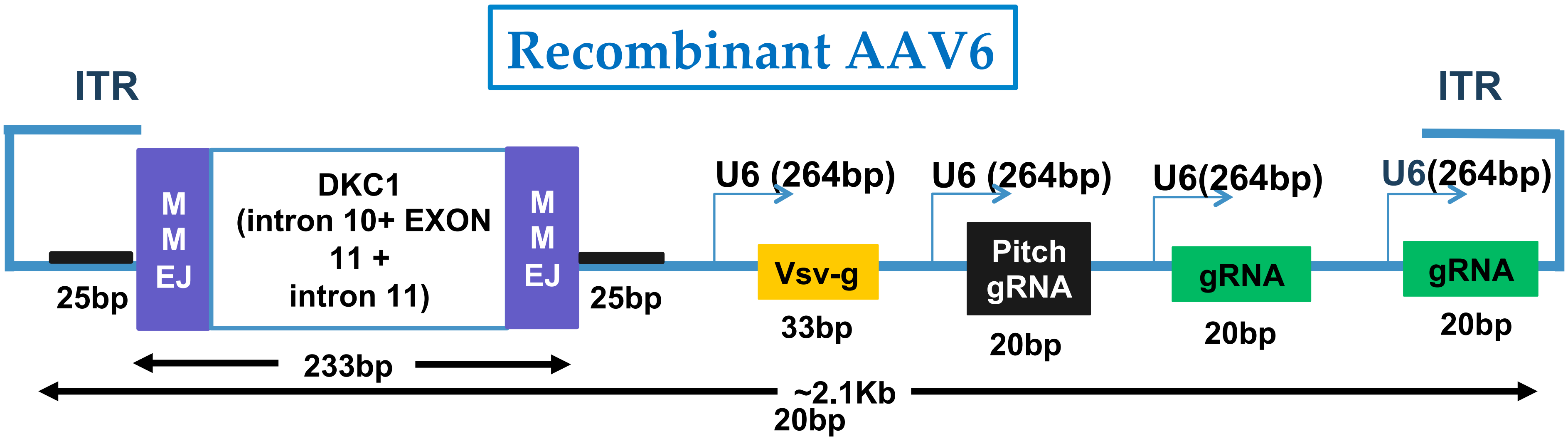
AAV6 Vector +AAV6 Cas9 + CRIS-PITCh

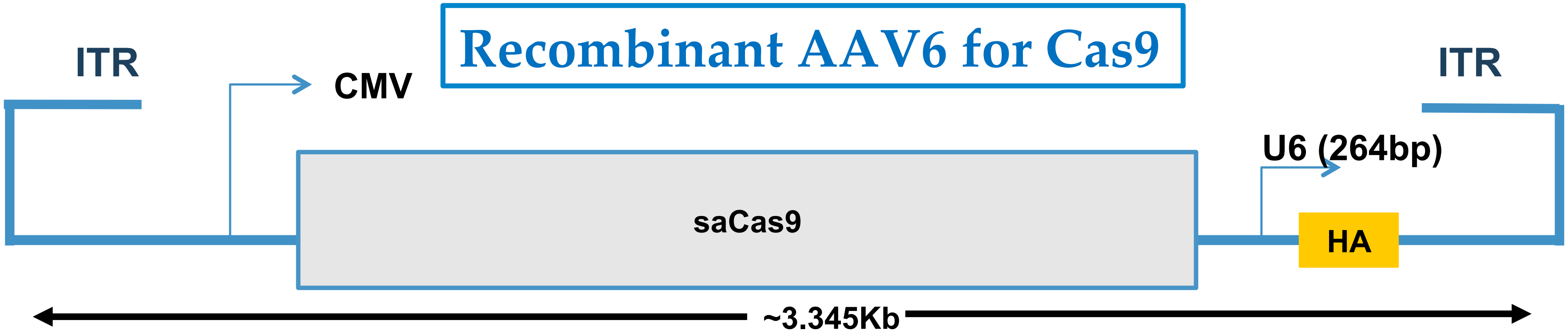
↙ ↘

iPS Hematopoietic Progenitors → CD34+ Humanized Mice

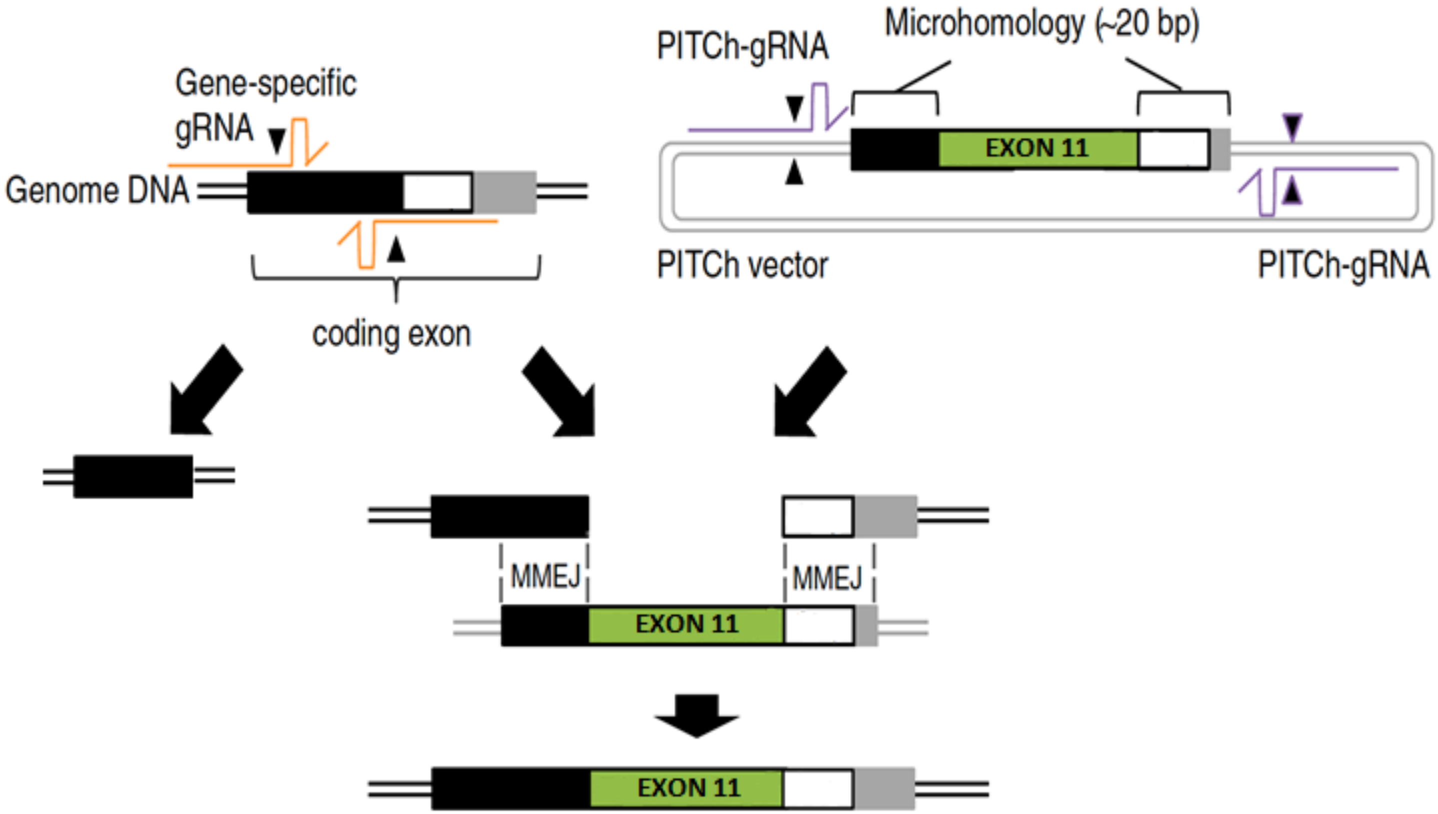
HPSC → ?

What is the System of Delivery?





CRIS-PITCh

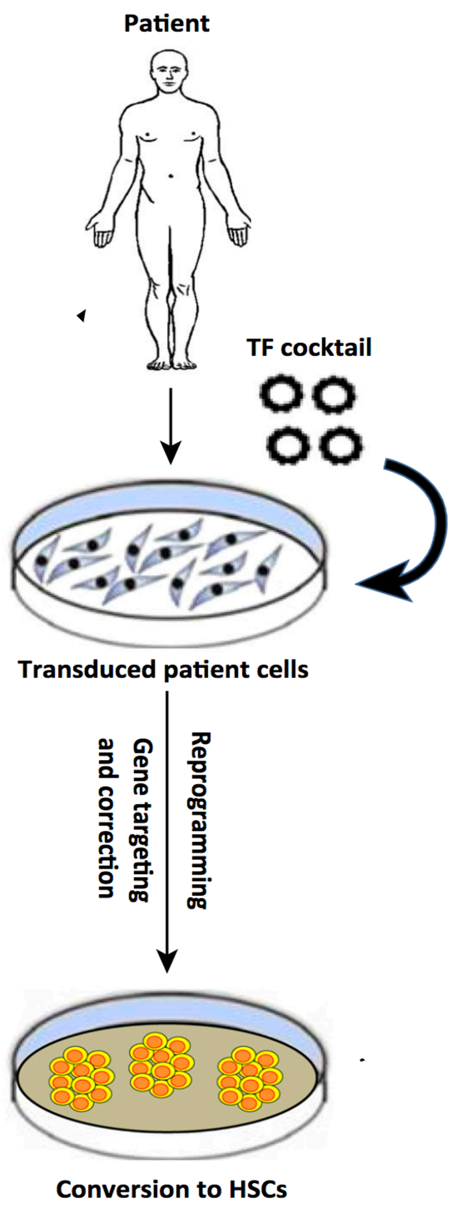


Tetsushi Sakuma *et al.*, Nature Protocol, 2016

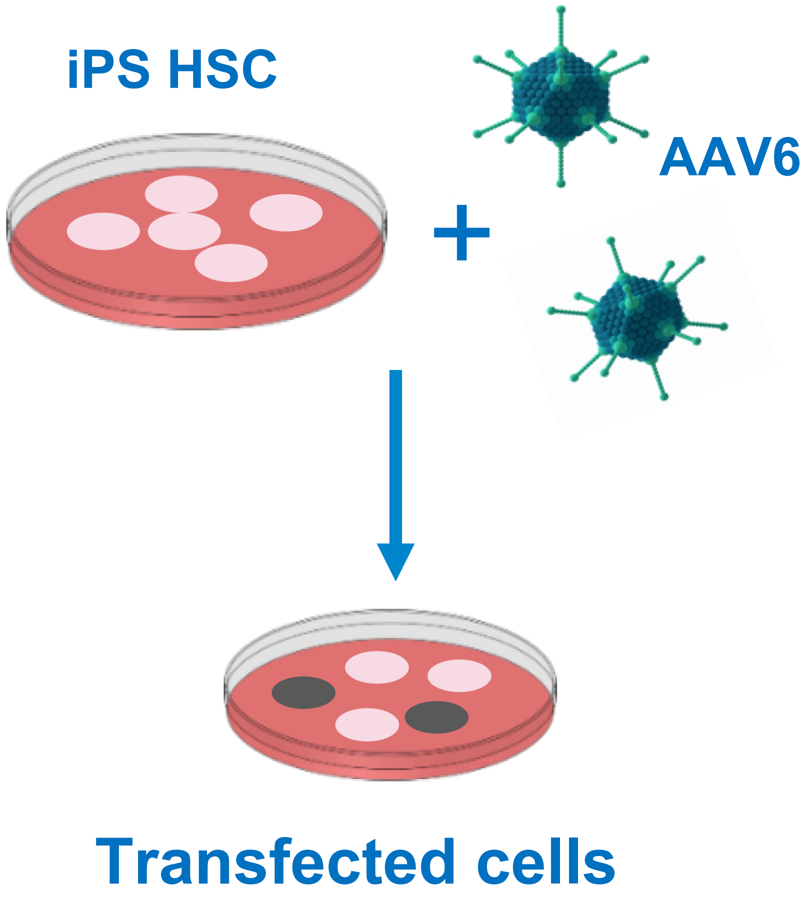
How to make iPS?

- Yamanaka factors to patient's fibroblasts
- To obtain HSCs: CDX4, HoxA9, ERG, RORA, SOX4, MYB
- Selection of HPSC with FACS

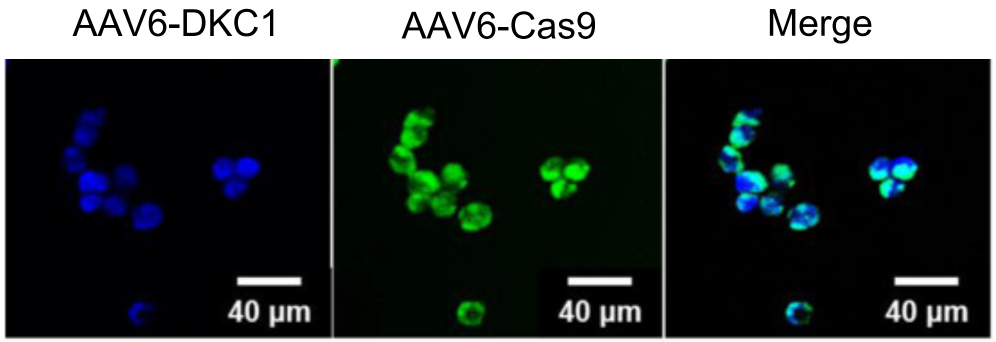
Michael G. Daniel *et al.*, Trend cells Biol, 2016.



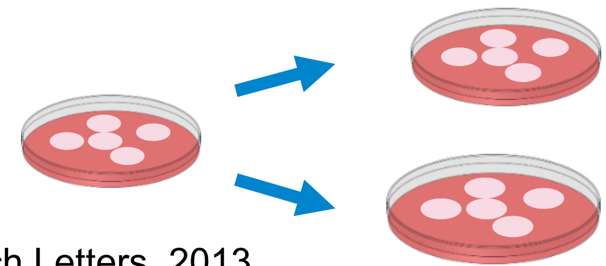
Transfection of iPS with our AAV6



Selection with FACS and titer calculation with ELISA



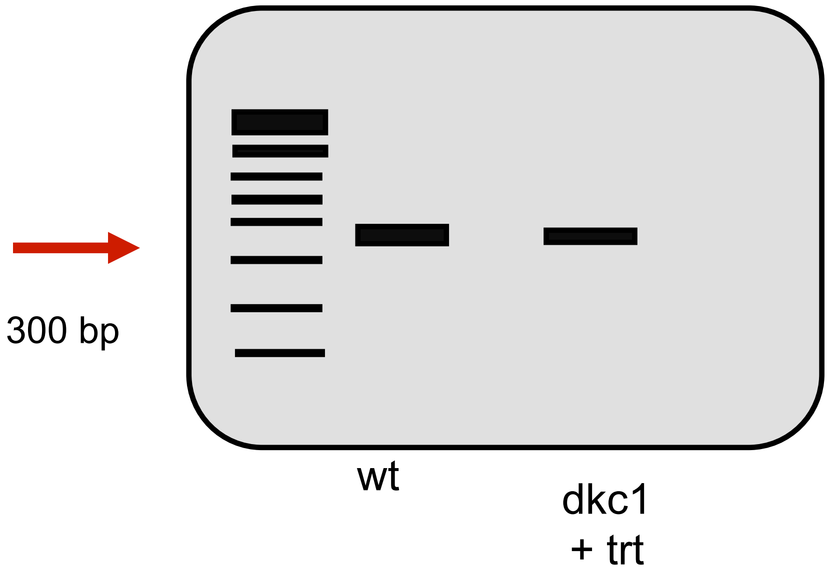
Clonal population



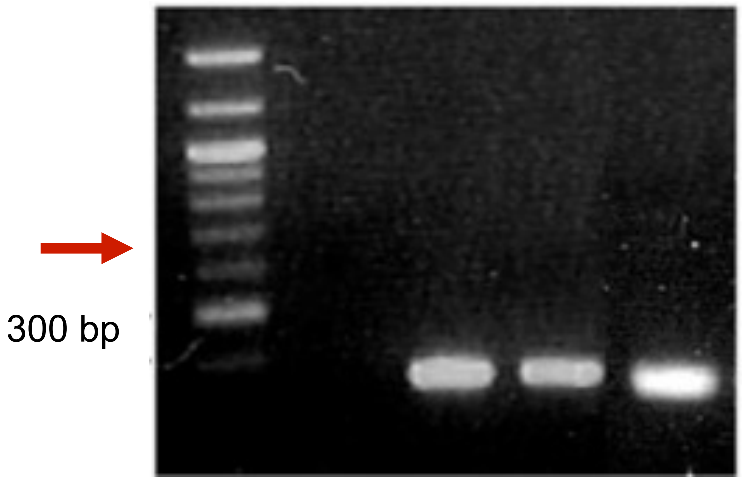
Kim B. *et al.*, Nanoscale Research Letters, 2013

Is the Integration Successful?

1. PCR to check the integration

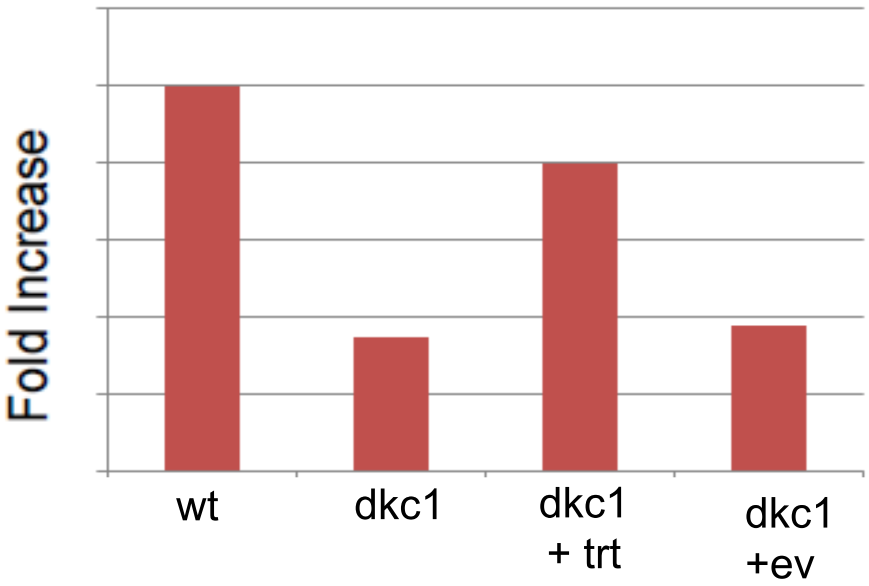


2. PCR for checking off-targets

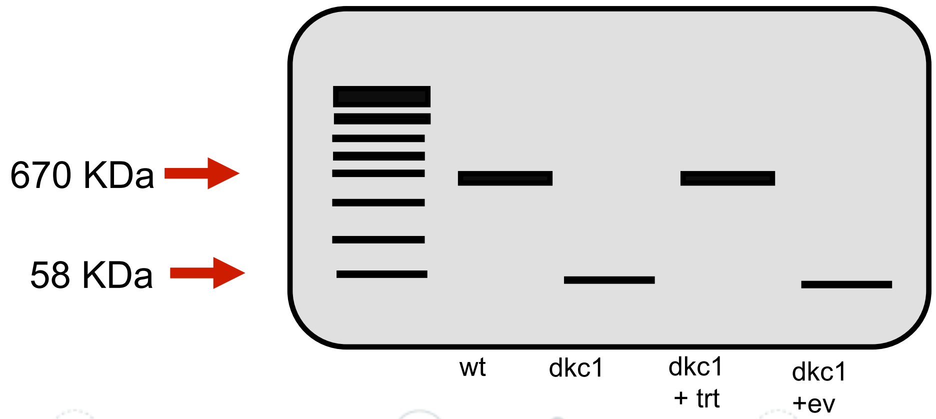


Is the Gene Functional?

3. qRT-PCR to verify the mRNA production

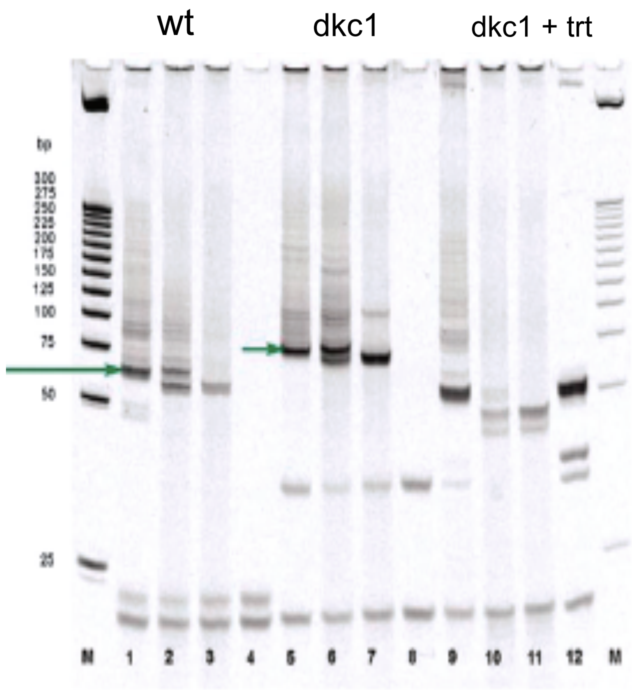


4. Co-IP to check the binding between DKC1 and telomerase complex

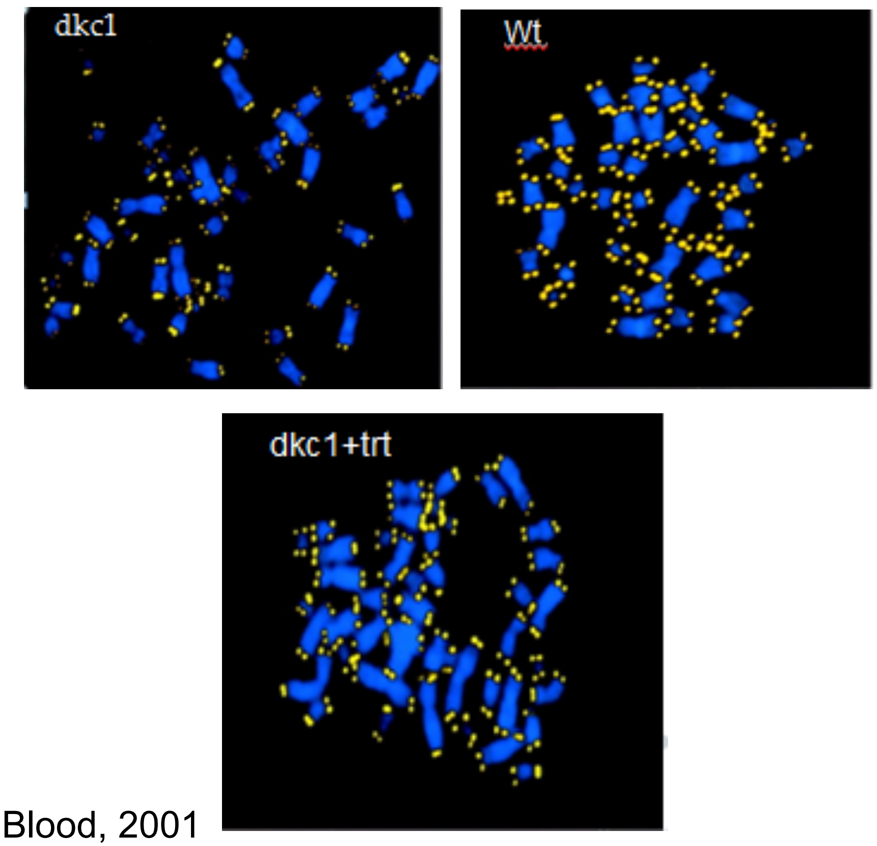


Is the Telomerase Working?

5. TRAP assay to check the functionality of the telomerase

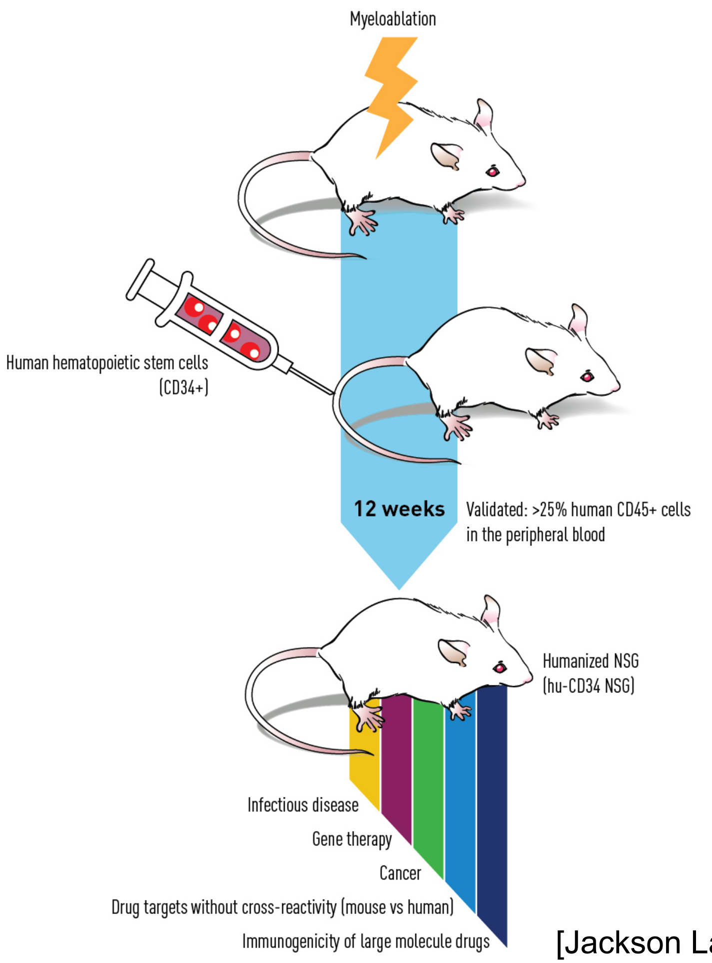


6. Q-FISH to check the lenght of telomeres



Rufer N. *et al.*, Blood, 2001

CD34+ Humanized Mice



- **Engraftment is stable for over one year without graft-versus-host disease.**

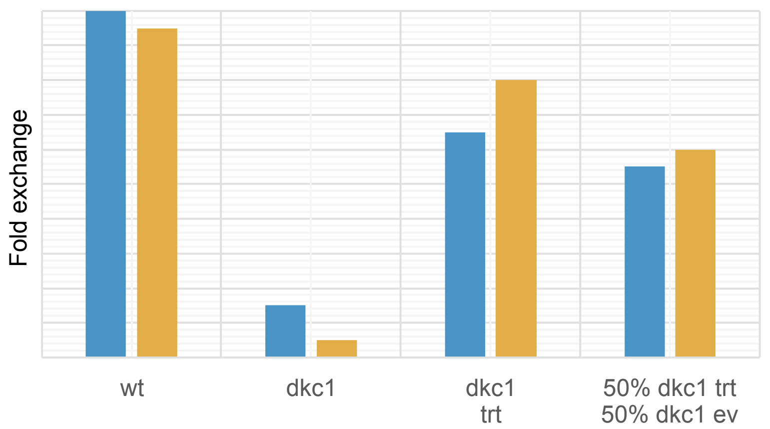
- **$CD4^+$ and $CD8^+$ T cells are present in circulation and other tissues**
  [Shultz et al. J Immunology 2005]

- **Good platform for screening efficacy of genetically modified stem cells**
  [Kitchen et al., 2012.]

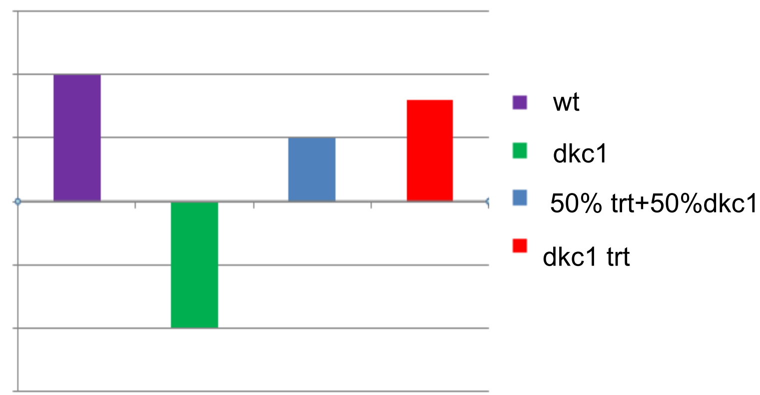
[Jackson Laboratory, (2017). Available at: http://www.jax.org/jax-mice-and-services/in-vivo-pharmacology/humanized-mice/cd34]

What are the outcomes of the transplantation?

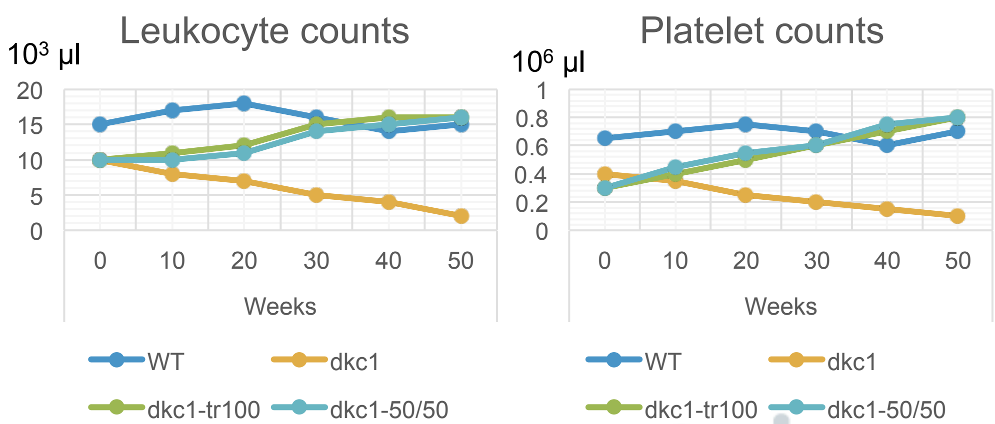
1. Blood HT-qFish



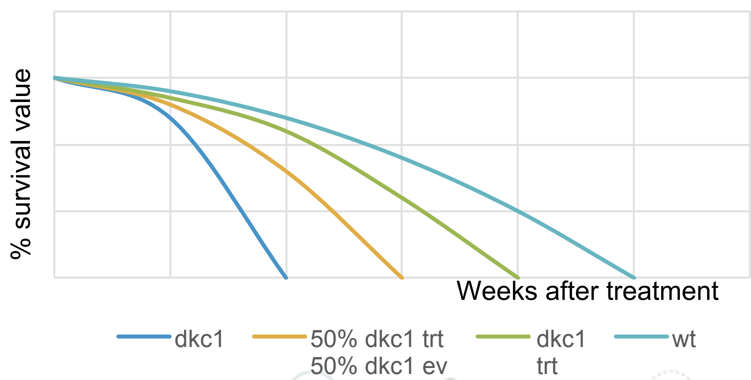
2. Cumulative cell population increase



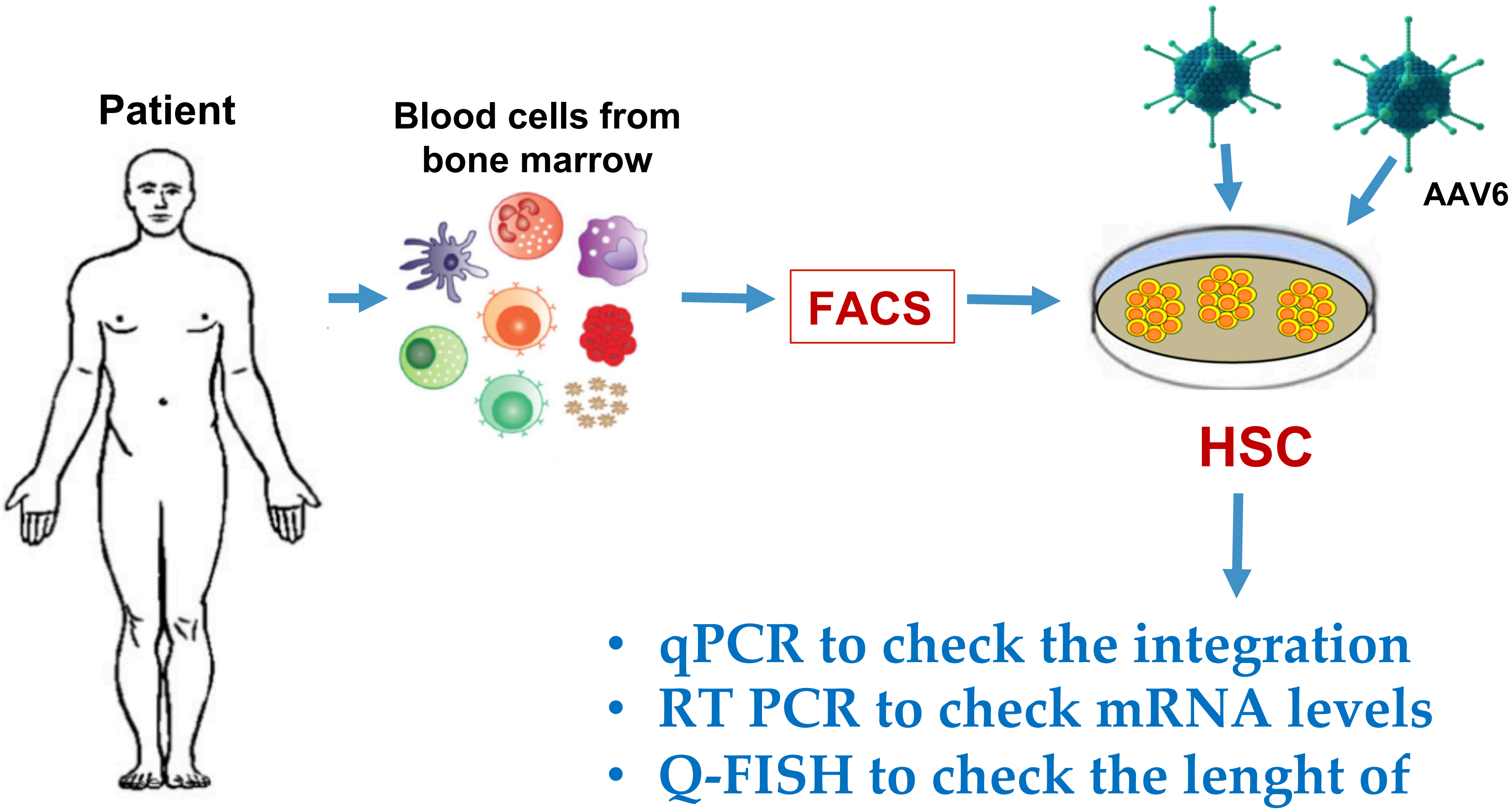
3. Blood withdrawals to check HPCs recovery



4. Mice survival

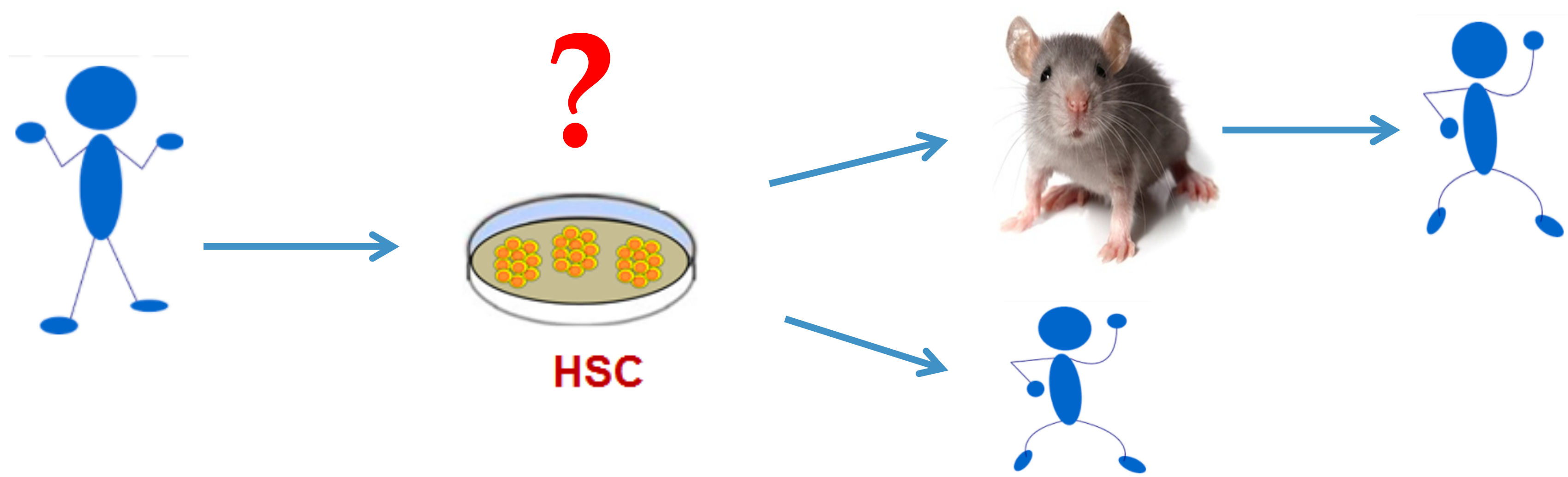


HSC from patients and controls



- qPCR to check the integration
- RT PCR to check mRNA levels
- Q-FISH to check the lenght of telomeres

Future Perspectives



- **If** the system of delivery and gene-editing works
- **If** the HSC from the patient are able to grow, allowing the minimum manipulations required

Clinical Trial

Pitfalls and Solutions

Pitfall:	Solution:
Low efficiency of transfection	Increase the amount of AAV6 or use another serotype
Inability to grow and expand HSC	Try with another medium
CRISPR/Cas9 causes off-target insertions	Design more efficient gRNAs
Problems with the modification of the PAMs in the transgene	Change the sequence of the gRNAs recognize in the transgene

Timescale, materials and cost of the project

Simplicon™ RNA Reprogramming Kit (OKSG) – Sigma-Aldrich	€ 1229
Pluripotent Stem Cell Culture Media – Sigma-Aldrich	€ 141
Hematopoietic Stem Cell Reagents – Sigma-Aldrich	€ 235
KiCqStart® One-Step Probe RT-qPCR ReadyMix™ – Sigma-Aldrich	€ 631
AccuTaq™ LA DNA Polymerase High fidelity Taq enzyme – Sigma-Aldrich	€ 215
CD34+ Humanized Mice - The Jackson Laboratory X 20	€ 2678
AAV6-CMV-Null Titer: 1x1013 GC/ml – Vector Biolabs	€ 495
Custom Primer, Value DNA Oligo – Thermo Fisher	€ 54
pCRIS-PITChv2-FBL plasmid - Addgene	€ 65
AAV Transduction Kit – 50 reactions – Antiboies-online.com	€ 885
H-3 DYSKERIN ANTYBODY (sc-373956) - Santa Cruz Biotechnology Co.	€ 285
Pierce™ Magnetic RNA-Protein Pull-Down Kit	€ 668
Stemline® Hematopoietic Stem Cell Expansion Medium – Sigma-Aldrich	€ 236
FISH Tag™ DNA Multicolor Kit, Alexa Fluor™ dye combination - Thermo Fisher	€ 662
HA tag (I9) Antibody + VSV-g (E11) Antibody – Delta Biolabs	€ 380

- **Time of the project: 24 months**
- **Cost per year: € 15.000**

+ Additional costs from basic lab manteinance and materials

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