SAPIENZA
UNIVERSITÀ DI ROMA

LM Genetica e Biologia molecolare nella Ricerca di Base e Biomedica **aa 2014/2015**

Gene Therapy **Prof. Isabella Saggio**

SCID AND GENE EDITING

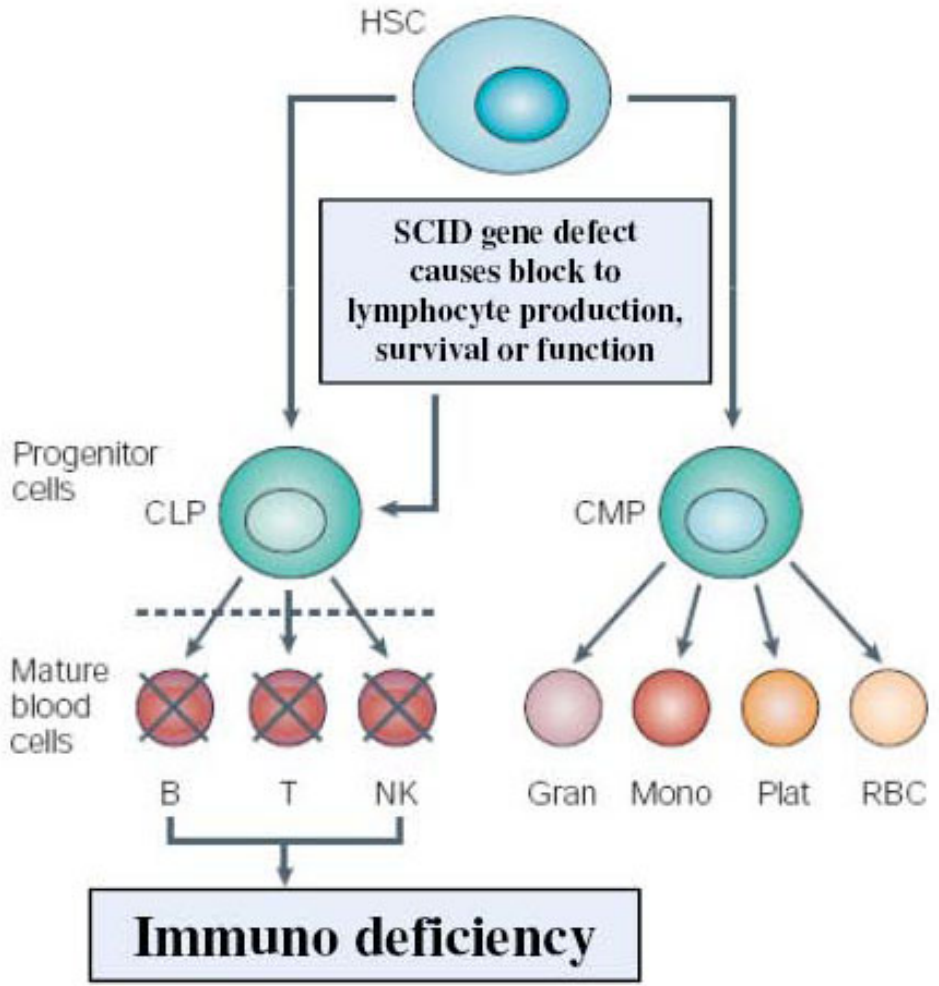
Students:
Del Giudice Simona
Parisi Sofia
Fioretti Elena
Larivera Simone

Tutor
La Torre Mattia

SCID "Severe Combined Immunodeficiency"

adapted from: http://www.geneticsandsociety.org/article.php?id=3840

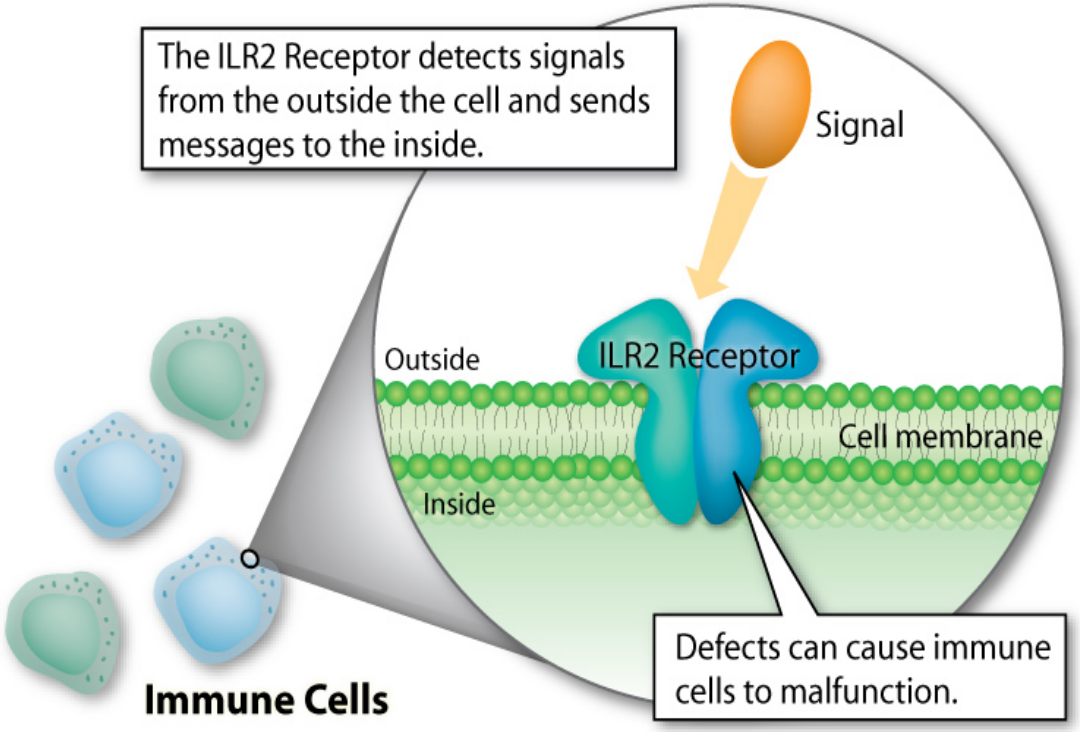
Group of rare and lethal conditions in which the infants die from an array of infections associated with a lack of lymphocytes in the blood.



adapted from: http://www.saggiolab.com/who-else-for-students/

X-Linked SCID (50% of all SCID cases)

X- SCID IS CAUSED BY INACTIVATING MUTATIONS IN IL2RG GENE



adapted from: http://learn.genetics.utah.edu/content/disorders/singlegene/scid/

- IL2RG gene mapped to chromosome Xq13.1
- IL2RG gene encodes the **interleukin 2 receptor common gamma chain**
- gamma c = common subunit of different interleukin receptors involved in growth and differentiation of lymphocytes.

IL2RG mutant → absence of signal cytokine activation → **X-SCID**

1°Gene Therapy clinical trial for X-SCID

Cavazzana-Calvo et Al. 2000

10 children were treated with Ex vivo gene therapy. Transduction of CD34+ bone-marrow cells, using MoMLV for delivering wt IL2RG gene.

Side effects

Genotoxicity of gamma retroviral integration into the genome

Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1

Salima Hacein-Bey-Abina,[1,2] Alexandrine Garrigue,[2] Gary P. Wang,[3] Jean Soulier,[4] Annick Lim,[5] Estelle Morillon,[2] Emmanuelle Clappier,[5] Laure Caccavelli,[1] Eric Delabesse,[6] Kheira Beldjord,[7,8] Vahid Asnafi,[7,8] Elizabeth MacIntyre,[7,8] Liliane Dal Cortivo,[1] Isabelle Radford,[8] Nicole Brousse,[9] François Sigaux,[4] Despina Moshous,[10] Julia Hauer,[2] Arndt Borkhardt,[11] Bernd H. Belohradsky,[12] Uwe Wintergerst,[12] Maria C. Velez,[13] Lily Leiva,[13] Ricardo Sorensen,[13] Nicolas Wulffraat,[14] Stéphane Blanche,[10] Frederic D. Bushman,[3] Alain Fischer,[2,10] and Marina Cavazzana-Calvo[1,2]

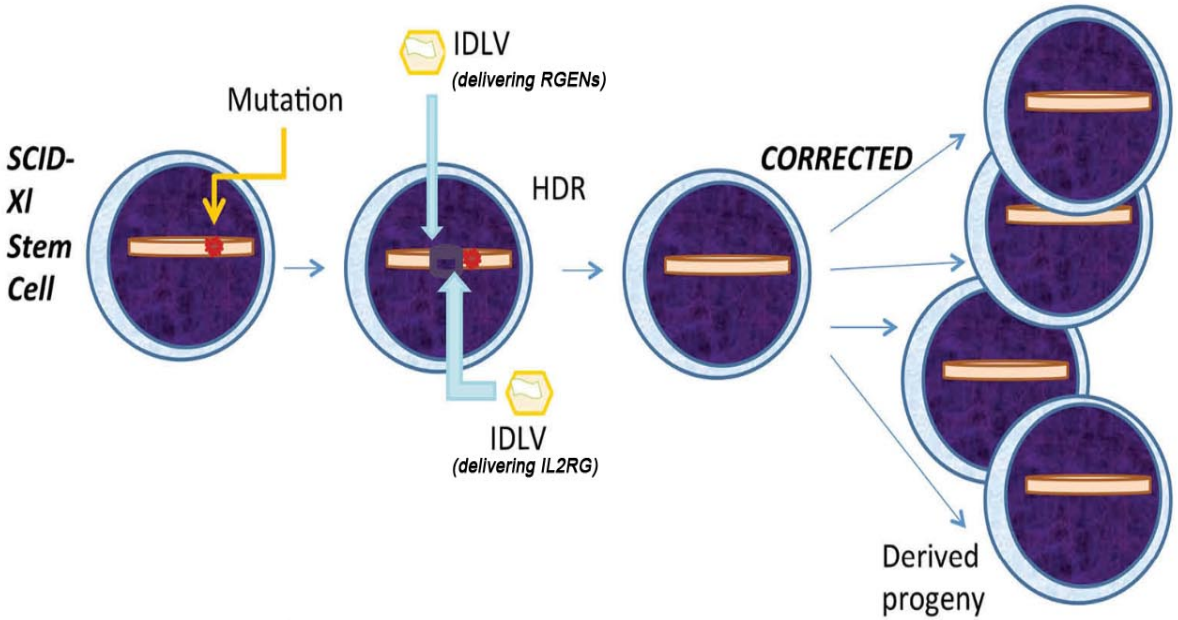
2008. J Clin Invst 118(9):3132.

Main issue:
Insertional Mutagenesis

OUR PROPOSAL

To overcome the insertional mutagenesis we propose:

Using gene editing tool RGENs (RNA guided engineered nucleases) to reach site-specific integration of IL2RG wt gene into "safe harbor" (AAVS1 locus) in human HSCs.

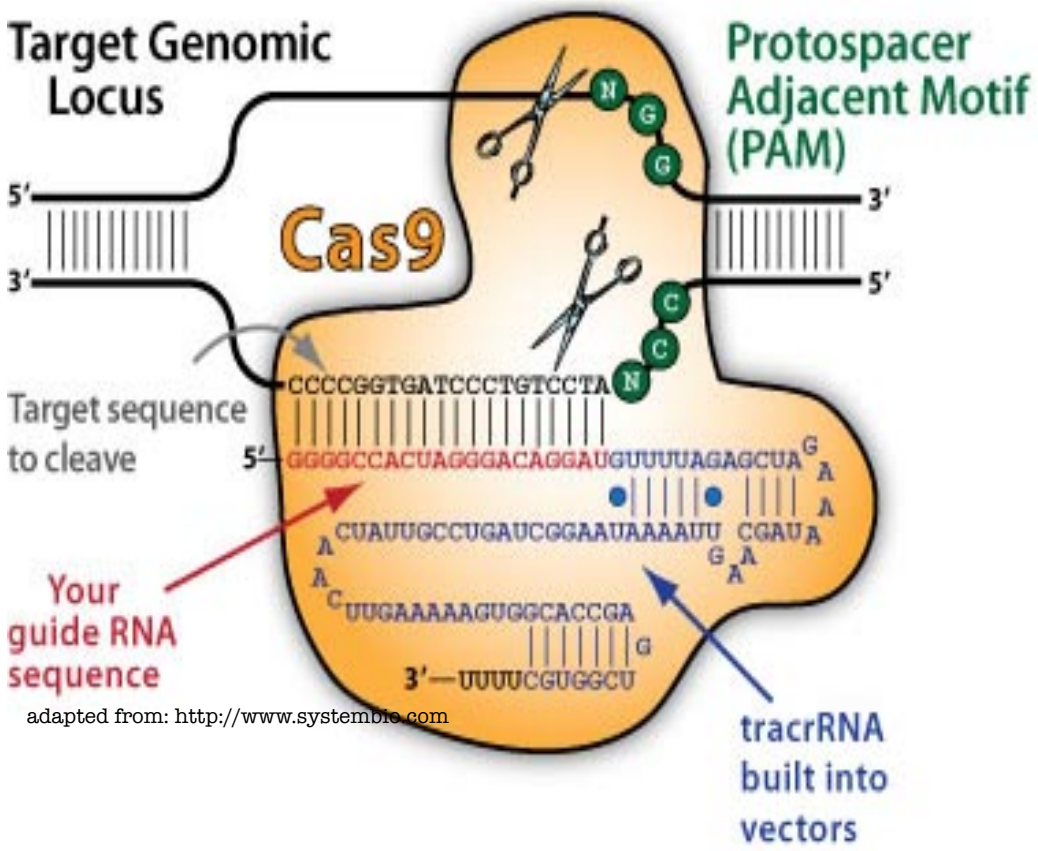


Transduction of X-SCID hHSCs with IDLVs vectors for delivering wild-type IL2RG gene and Cas9/Crispr system.

Cas9/CRISPR system allows a site-specific integration of the therapeutic gene in AAVS1 locus to **rescue immune system function.**

adapted from: http://www.hematology.org/Thehematologist/Diffusion/3166.aspx

RNA - guided engineered nucleases (RGENs)



- Derived from bacterial CRISPR-Cas 9 system
- Produce DSBs and enhance HDR efficiency in a specific way
- Work as monomer
- Low cytotoxicity
- Convenient to produce

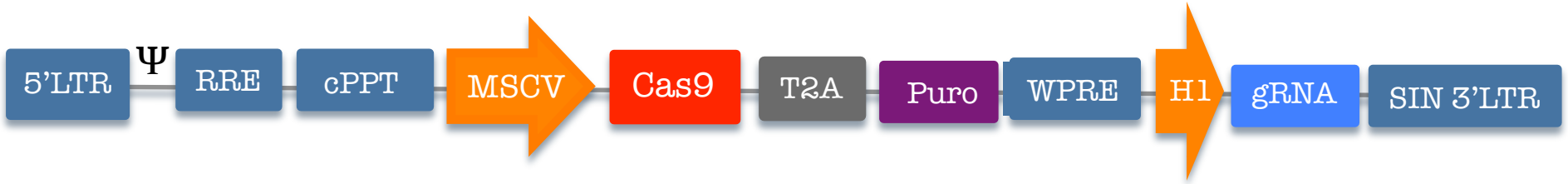
gRNA directs Cas9 nuclease on a 20 bp complementar DNA sequence. Cas9 perfoms DSBs that trigger endogenous DNA repair system resulting in a targeted genome modification.

IDLVs VECTORS

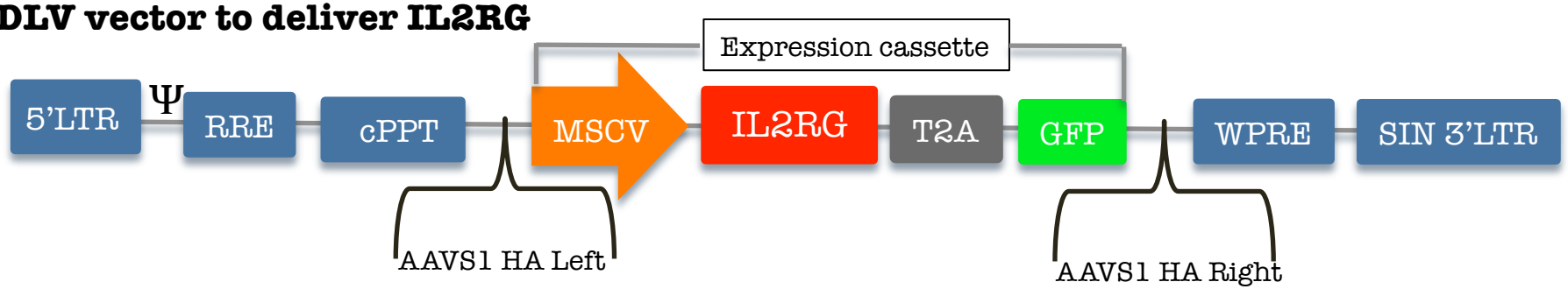
FEATURES

- HIV-1 based lentiviral vector (third generation) carrying a class I IN mutation in the D64V region of the catalytic domain, resulting in an **inhibition of vector's integration**.
- Possibility to provide a **site specific integration** in the genome **through RGENs**.
- Possibility of use in ex vivo experiment without forcing cells replication.

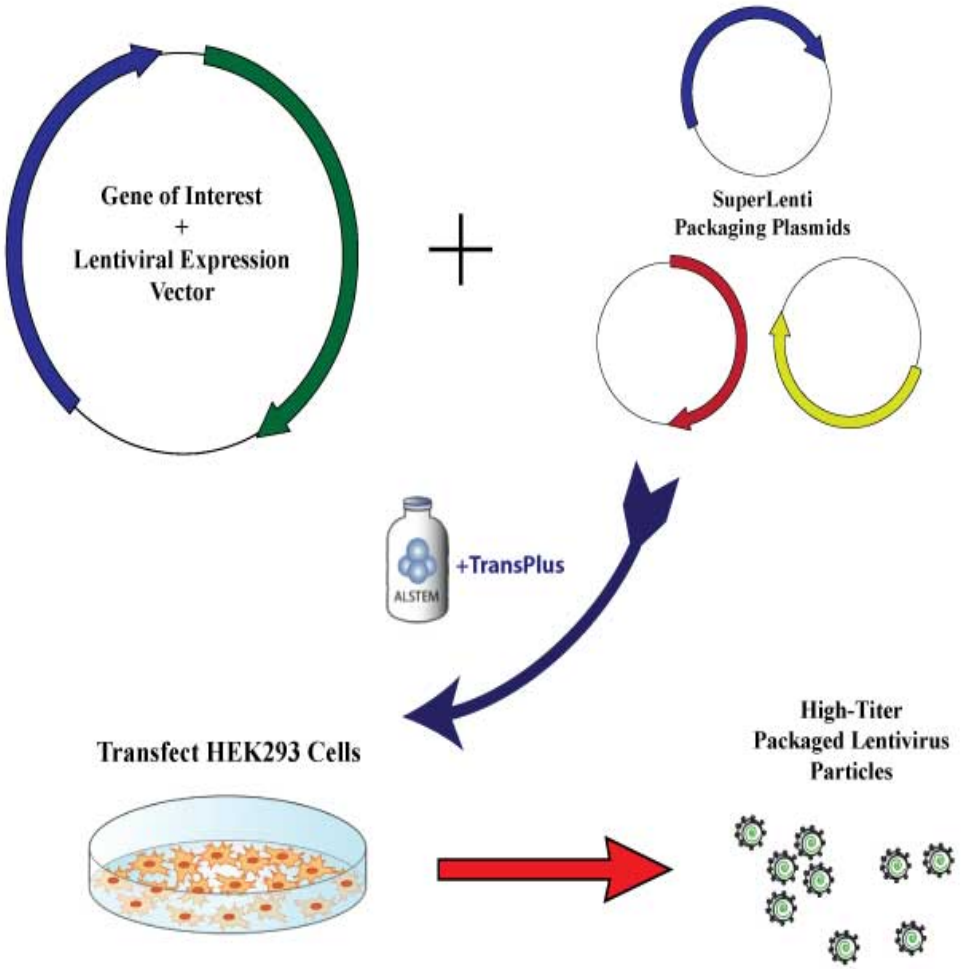
IDLV vector to deliver Cas9-CRISPR system



IDLV vector to deliver IL2RG



IDLVs PRODUCTION



Adapted from http://www.alstembio.com/virus-production/Lentiviral-packaging-mix

Cells HEK 293T are transiently transfected by:

1. Expression plasmid
2. Packaging plasmid
3. Pseudotyping plasmid
4. Plasmid containing *rev* gene.

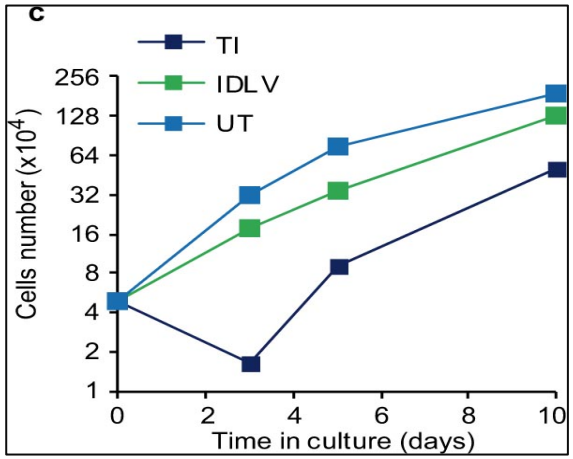
Ultra – centrifugation to obtain supernatant containing the lentiviral particles.

p24ELISA assay to estimate the titer of the lentiviral vectors within supernatant.

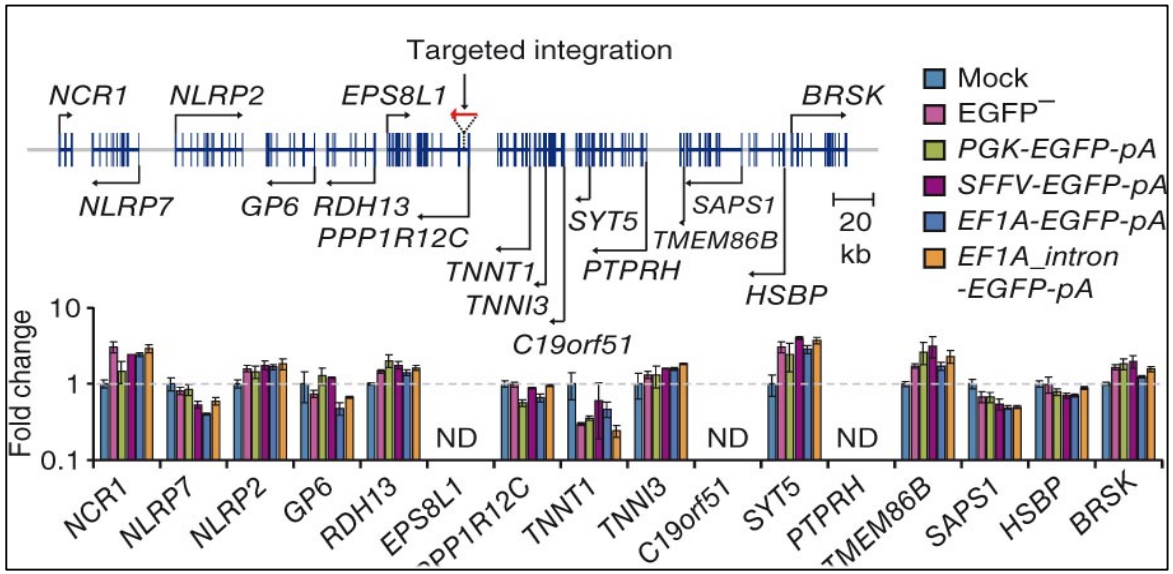
AAVS1 Locus as "Safe Harbor"

AAVS1 is a common integration site of the human non pathogenic adeno-associated virus, found between exon 1 and intron 1 of protein phosphatase 1 regulatory subunit 12C (PPP1R12C) gene, located in chromosome 19.

Different studies showed the **safe, stability and robustness of transgene expression** after insertion in *AAVS1* of human stem cells (iPSs, hHSCs).



Luigi Naldini, Pietro Genovese et AL., nature13420, 2014



DOI:10.1038/NMETH.1674

Transgene's insertion at the AAVS1 locus revealed **no upregulation** in gene expression of flanking genes.

IN VITRO EXPERIMENT

IN VITRO MODEL:

-Human Hematopoietic Stem Cells (HSCs) bone marrow-derived from X-SCID infants

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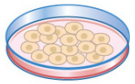
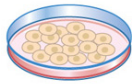
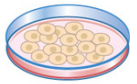
1. TRANSDUCTION

	UNT	IDLV Cas9 + empity IDLV	IDLV Cas9 + IDLV IL2RG
hHSCs			

2. SELECTION OF POSITIVE TRANSDUCED CELLS USING PUROMYCIN

3. ISOLATION AND PROPAGATION OF SINGLE COLONIES

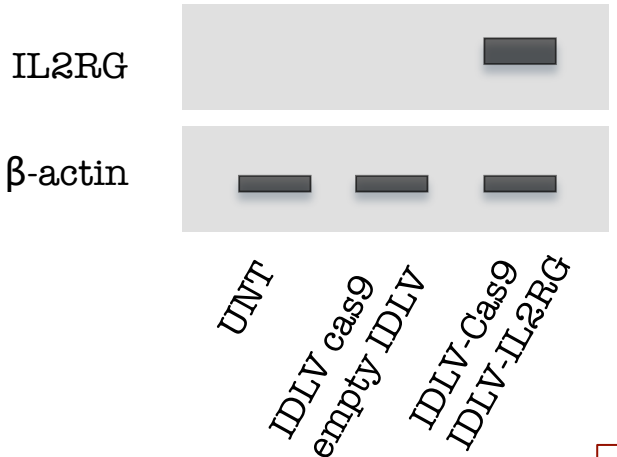
4. INSERTION'S SITE CONTROL THROUGH LAM - PCR

5. LAM PCR POSITIVE CELLS ARE PROPAGATED AS A CLONE

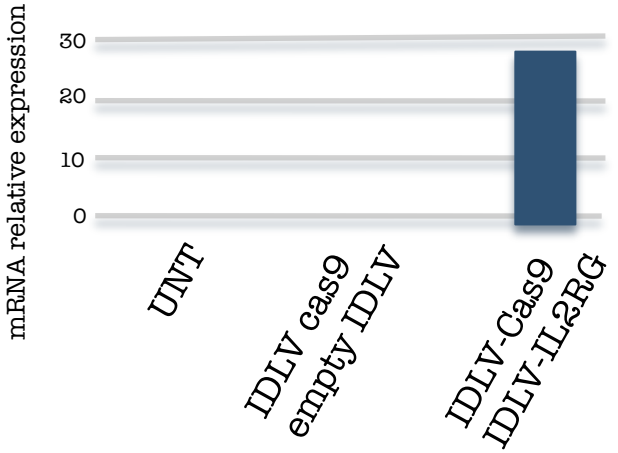
IN VITRO EXPERIMENT

IL2RG EXPRESSION CHECK

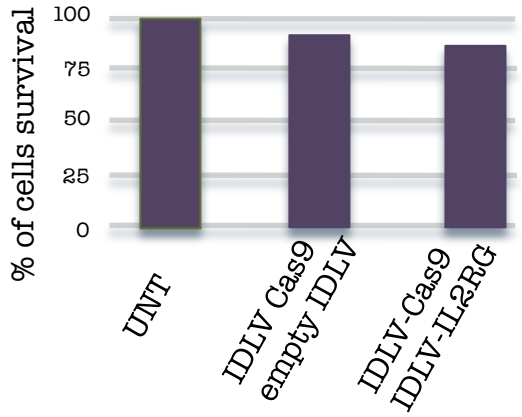
WESTERN BLOT for IL2RG in hHSCs



RT qPCR for IL2RG in hHSCs



VIABILITY ASSAY (MTT)



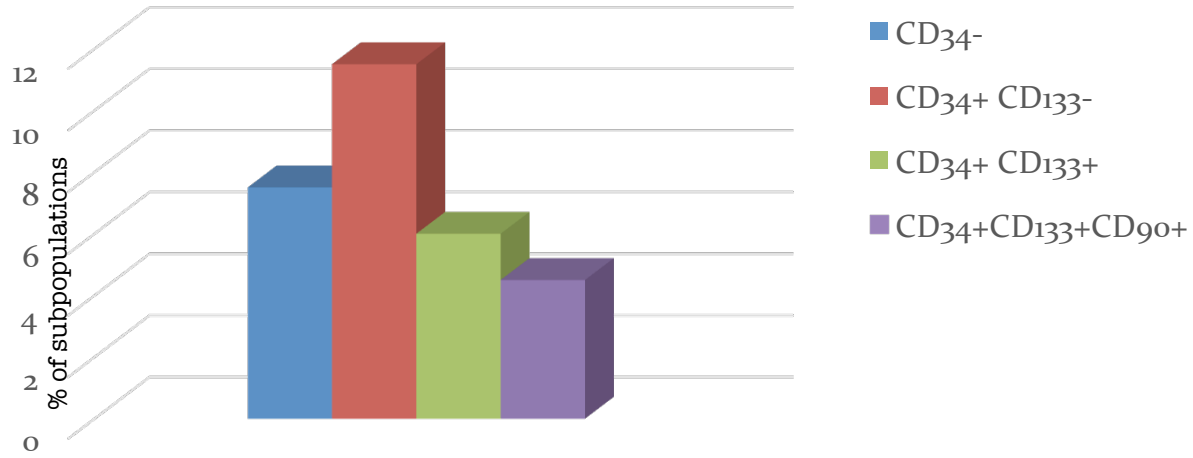
IN VITRO EXPERIMENT

FUNCTIONAL ASSAY

Induction of hHSCs' differentiation adding specific growth and differentiation factors.

FLOW CYTOMETRY

using different surface markers to evaluate the presence of mature differentiated subtypes of lymphocytes



Adapted from: Luigi Naldini, Pietro Genovese et AL., nature13420, 2014

IN VIVO EXPERIMENTS

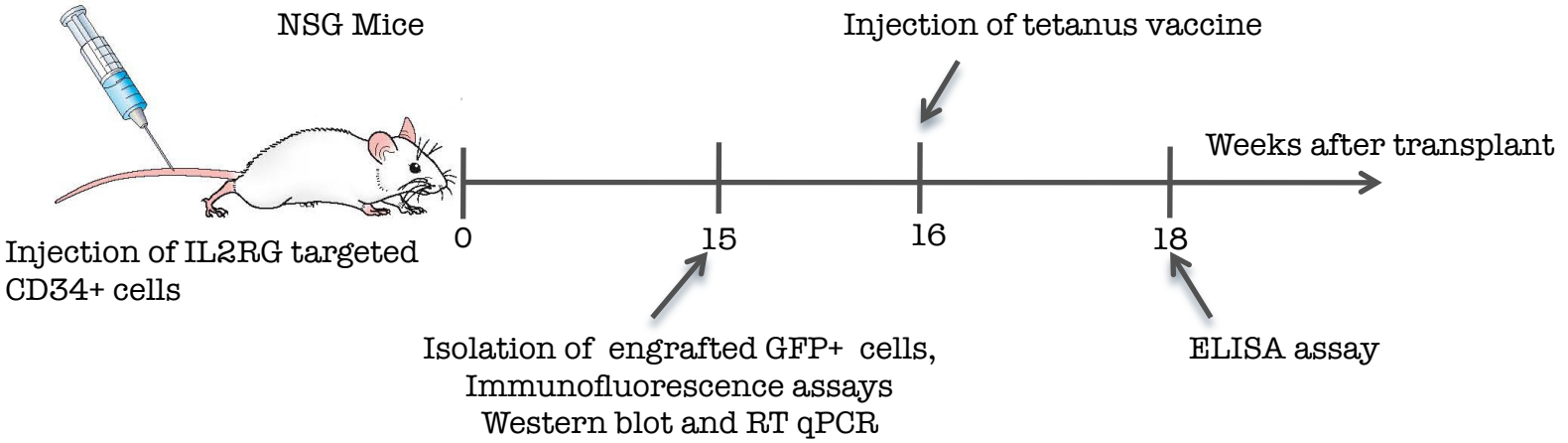
IN VIVO MODEL:

NOD SCID gamma (NSG) Mice

- Albino, viable, fertile, normal size, do not display any abnormalities
- Lack mature T cell, B cells and NK cells
- Deficient in cytochine signaling
- Abnormal immune system organ morphology
- Resistant to lymphoma development
- Support engraftment of hHSCs
- Median survival time 89 weeks

http://jaxmice.jax.org

IN VIVO EXPERIMENTS



1. Transplantation of gene-targeted CD34+ cells in NSG mice

2. Isolation of engrafted GFP + cells from mice's bone marrow and blood by FACS

3. Characterization of engrafted GFP + cells

Evaluate the repopulation of immune system

IMMUNOFLUORESCENCE of GFP + cells

To control IL2RG expression

WESTERN BLOT of GFP + cells

RT qPCR of GFP + cells

IN VIVO EXPERIMENTS

Does our strategy work?

↓

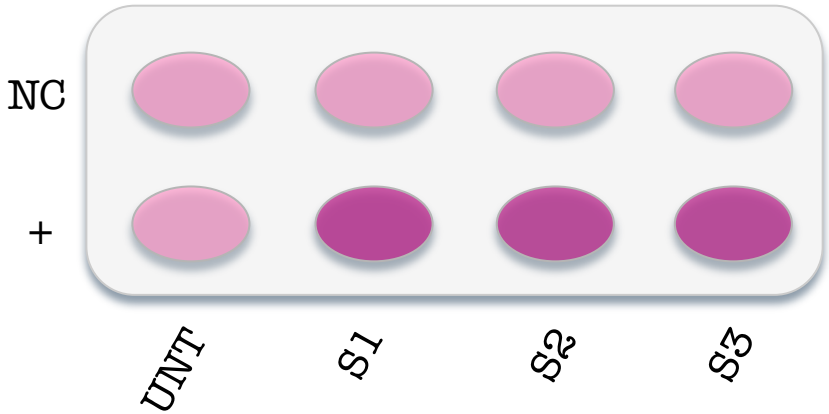
Do Mice rescue a normal phenotype with functional immune system ?

↓

Evaluation through injection of tetanus vaccine

FUNCTIONAL ASSAY

Perform an ELISA assay to verify the presence of anti-tetan antibodies



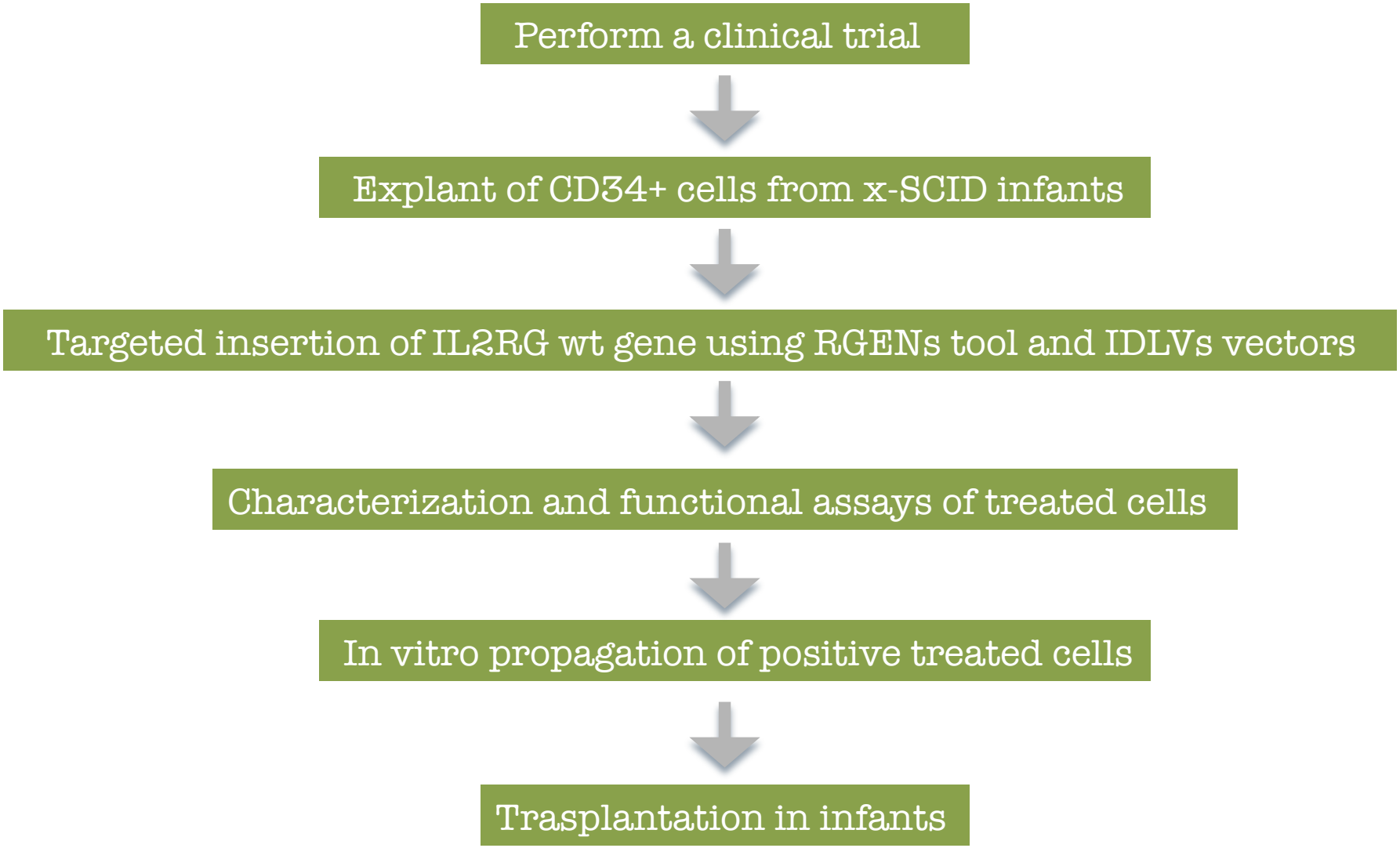
NC = negative control

+ = test positive

S = sample

UNT = untreated

FUTURE PERSPECTIVES



COSTS & TIME

• LENTI – Smart ™ NIL (InvivoGen) + Additional materials *	
• LAM PCR:	
- Taq DNA polymerase (Qiagen)*	
- dNTPs (Fermentas)*	
- Oligonucleotides and primers MWG Biotech*	
- Magnetic particles: Dynabeads M---280 Streptavidin (Dynal) (Lifetechnologies)	452 $
- Kilobase binder kit (Dynal) (Lifetechnologies)	417 $
- Klenow polymerase (Roche)	109 $
- Hexanucleotide mixture (50 reactions) (Roche)	88 $
- Restriction endonuclease(s) and incubation buffer(s) (New England Biolabs)*	
- Fast - Link DNA ligation kit (Epicentre)	142 $
- T4 DNA Ligase (New England Biolabs)	62 $
- Spreadex EL1200 precast gel (Elchrom Scientific)*	
- QIAquick PCR purification kit (Qiagen)*	
- DNA extraction kit (Qiagen)	
• TaqMan (Applied Biosystems) 111$	
• NSG Mice (The Jackson Laboratry)	161$ per mouse
• Stabulation	700 $ per month
• Tetanus' Vaccine (Sanofi Pasteur MSD)	10,20 $ per dose
• ELISA Kit for Tetan's Vaccine (BioCompare)	247 $
• Anti – IL2RG Antibody host mouse (Sigma-Aldrich)	386 $
• Secondary Anti – Mouse Antibody (Sigma-Aldrich)	237 $
• Antibody for specific sufrace markers of HSCs Cells and their progeny host rabbit (Bioss) *	
• Secondary Anti – Rabbit antibody (Pierce)*	
• FACS Kit (BD Bioscience)*	
• MTT Cell Proliferation Assay Kit (1000 Assays) (Life technologies)*	

TIME: predict 30 months with an amount of 20000/30000$
the possibility of collaboration with other laboratories could decrease costs

*Contact distributor

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