WISKOTT ALDRICH SYNDROME GENE EDITING IN HUMAN CD34$^+$ CELLS USING TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES(TALENS)

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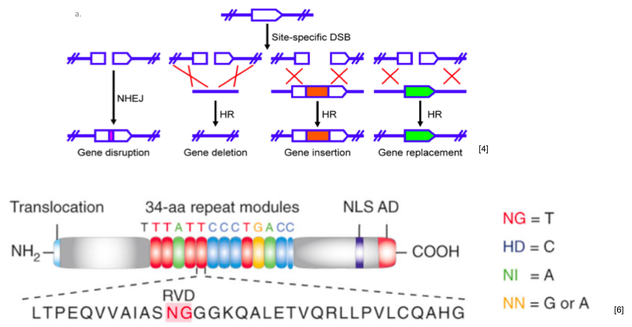
INTRODUCTION

Wiskott-Aldrich syndrome (WAS) is a rare and severe X-linked primary immunodeficiency (PID) caused by mutations in *WAS* gene coding for the WAS protein (WASp), a cytoplasmic protein, expressed exclusively in hematopoietic cells and involved in actin cytoskeleton reorganization. The disorder is characterized by micro-thrombocytopenia, eczema, recurrent infections, higher susceptibility to develop autoimmune manifestations and lymphoid malignancies. Allogeneic hematopoietic stem cell (HSC) transplantation is a highly effective curative option although limited by the availability of donors. Therefore, *ex vivo* gene therapy using autologous HSC could represent a valid option in patients without HLA-matched donors and severe clinical signs. So for that, several studies have been conducted using viral vectors. The first clinical trial using a gamma-retroviral (RV) vector showed restoration of WASp expression in multiple lineages and an improvement of the clinical condition of patients.[1] However, the occurrence of four cases of leukemia reported in 4 out of the 10 patients treated and other cases of insertional mutagenesis brought about by the use of RV vectors show the drawbacks of the treatment with such vectors.[2] On the other hand, self-inactivating lentiviral vectors (LV) may represent a substantial enhancement in terms of safety profile. Moreover, a clinical trial (phase I/II) using LV-transduced autologous CD34+ cells started in 2011[3] , but we can not rule out the possibility of insertional mutagenesis. To overcome this problem, an alternative approach using engineered nucleases for precise genome editing is proposed. We will use TALENs in a genome-editing strategy whereby we targeted a specific common *WAS* gene mutation (665C>T) to be edited.

Engineered nucleases: TALEN

Engineered nucleases are composed by sequence-specific DNA-binding domains fused to a nonspecific DNA cleavage module, enabling efficient gene modifications by introducing targeted DNA double-strand breaks (DSBs) that stimulate both, error-prone nonhomologous end joining (NHEJ) and homology-directed repair (HDR) DNA repair pathways. A TALEN consists on an engineered TALE repeatitive array fused to the FokI nuclease domain. Their targeting specificity is determined by a central repeatitive and conservative unit containing 33-35 amino acids with only two hypervariable residues (repeat variable diresidues, RVD) located at positions 12 and 13. These RVD determine the target nucleotide sequence, through the interactions between RVD: NN, NI, HD, and NG and the DNA bases: guanine, adenine, cytosine, and thymine, respectively.

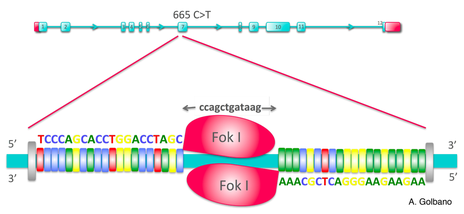


OBJECTIVES

1. **Target, construct and validate a WAS TALEN**
   - a. To target a WAS gene sequence for potential TALEN sites nearby 665 C>T mutation
   - b. To construct and validate an efficient TALEN
   - c. To identify TALEN on target activity and off target activity
2. **Test and select TALEN in vitro edited cells**
   - a. To design an oligonucleotide donor (ODN) containing the correct base and a floxed-PGK-puromycin cassette as a selection method
   - b. To select TALEN-edited cells
   - c. Test WASp expression on TALEN-edited cells
3. **Test in vivo correction**
   - a. To transplant TALENT edited cells into Rag2$^{-/-}$/gamma-c$^{-/-}$ mice
   - b. Test biodistribution, differentiation and engraftment of corrected CD34$^+$cells

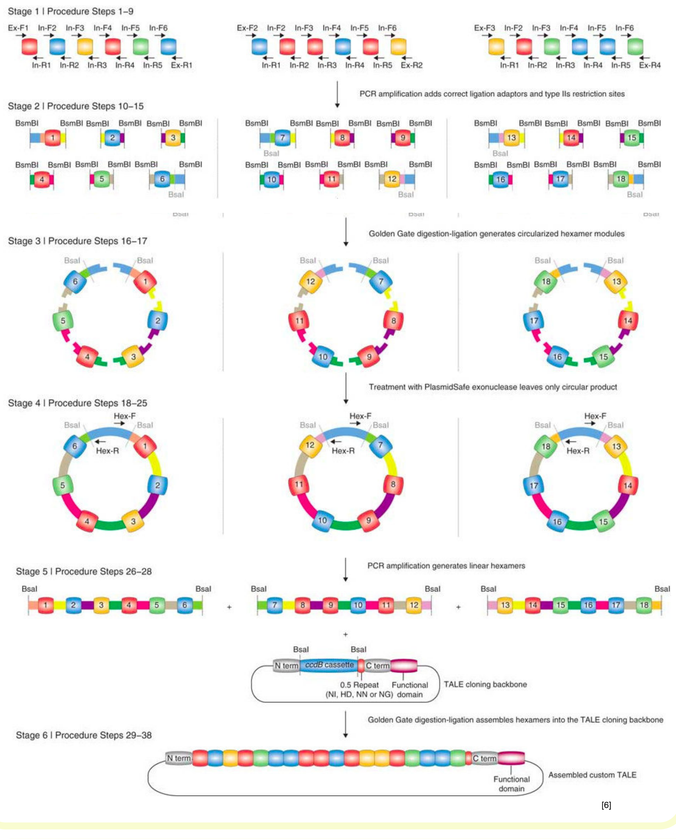
MATERIALS AND METHODS

1. Target a TALEN site. Using the TALENT Library Resource database [5] a TALENT target site was chosen, proximal to 665 C>T premature stop codon (PSC).

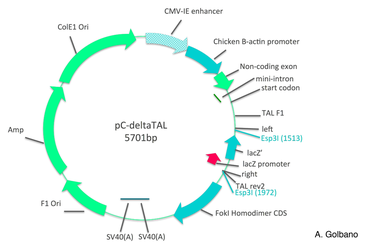


2. Generation of patients-specific nuclease.

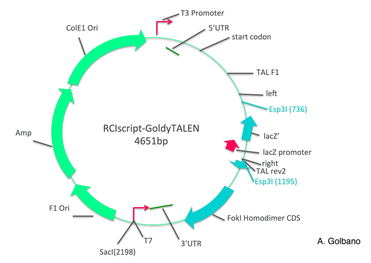
Using the Golden Gate assembly method.



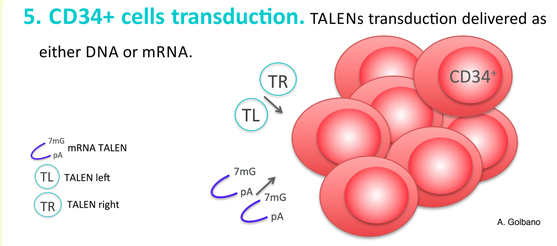
3. Cloning into GoldyTALEN mini-CAGGs promoter expression cassette. TALEN as DNA.



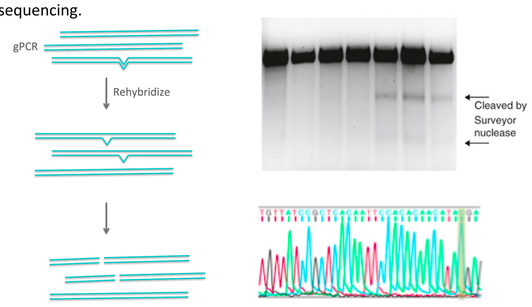
4. Cloning into RCIscript-GoldyTALEN vector. TALEN as mRNA.



5. CD34+ cells transduction. TALENs transduction delivered as either DNA or mRNA.



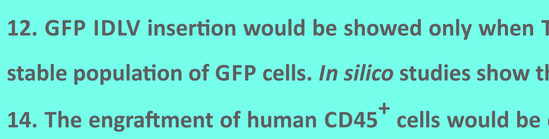
6. Surveyor nuclease assay. Genomic PCR (gPCR) is used to amplify the TALEN target region from a heterogeneous population of TALEN-modified and TALEN-unmodified cells, and the gPCR products are reannealed slowly to generate heteroduplexes. The reannealed heteroduplexes are cleaved by Surveyor nucleases, whereas homoduplexes are left intact. Confirmation by sequencing.



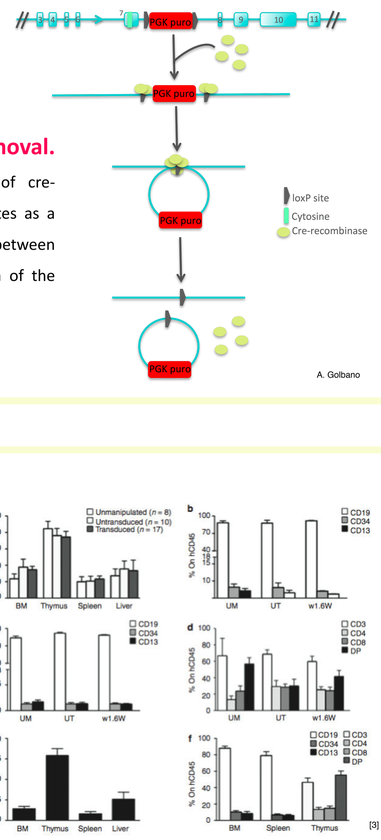
7. Corrective donor assembly. The double-stranded plasmid DNA donor is composed by *WAS* genomic sequences comprising a right arm (899 bp long), and a left arm (547bp long) that contains the 665 C>T mutation corrected. Left and right arms are cloned into a *floxed*-PGK-puromycin cassette, flanked by loxp sites and oriented such as it would be knoked into the intron between exons 7 and 8.

8. Selection. Deliveration of donor and TALENs as either plasmid DNA or mRNA to CD34$^+$ cells, selection in bulk using puromycin, sequestration into subpools, and finally, isolation and expansion of clones.

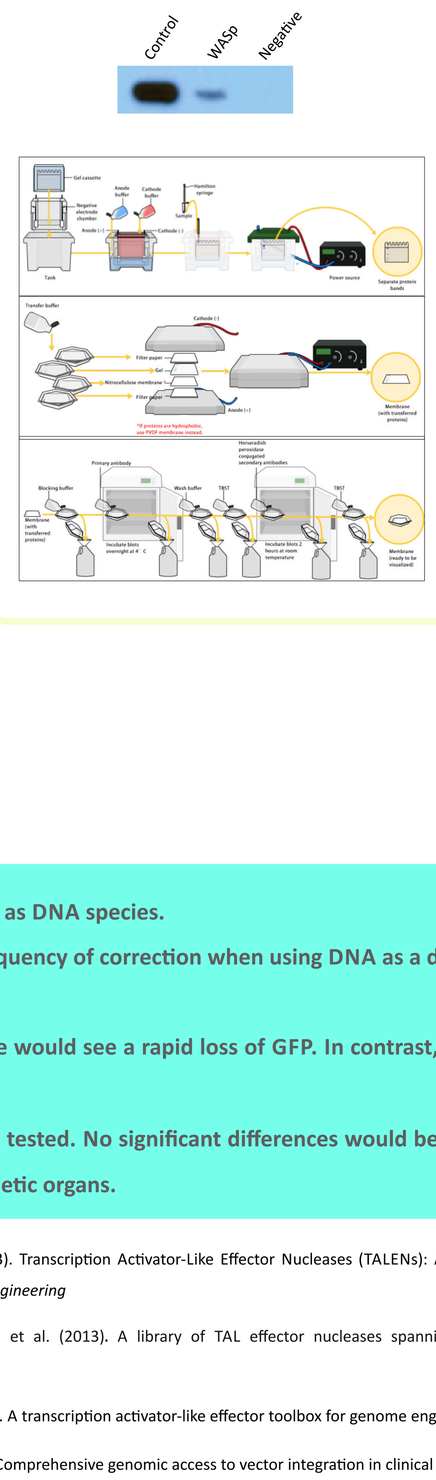
9. TALEN-edited cell screening. PCR analysis of both post-TALEN-treated subpools and clones, using the primers indicated with arrows. Confirmation by sequencing.



10. Puromycin cassette removal. Using Cre/loxP system. Expression of cre-recombinase, assembly at the loxP sites as a dimer. Induction of recombination between loxP sites as a tetramer and, excision of the floxed DNA sequence.

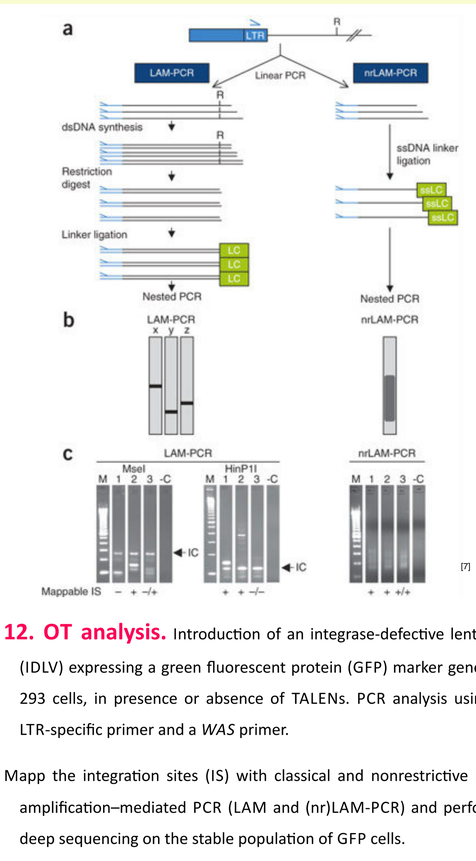


11. WASp expression analysis. Western Blot analysis in different cell lines derived from CD34$^+$cells, comparing TALEN-treated with untreated mutant cells.



12. OT analysis. Introduction of an integrase-defective lentivirus (IDLV) expressing a green fluorescent protein (GFP) marker gene into 293 cells, in presence or absence of TALENs. PCR analysis using an LTR-specific primer and a *WAS* primer.

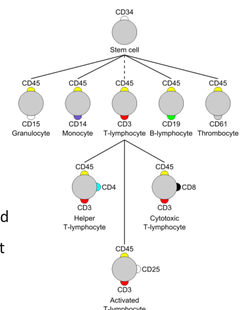
Mapp the integration sites (IS) with classical and nonrestrictive linear amplification–mediated PCR (LAM and (nr)LAM-PCR) and perform a deep sequencing on the stable population of GFP cells.



13. TALEN-edited CD34+ cells transplantation. Corrected CD34$^+$ cells are transplantated into Rag2$^{-/-}$/c$^{-/-}$ neonate mice, which had been sublethally irradiated . Control animals will be transplanted with the same amount of unmanipulated (UM) CD34$^+$ cells from helthy donors.

14. Biodistribution analysis of human derived cells.

Different organs (BM, thymus, spleen, and liver) will be analysed by flow cytometry using diferent cell markers, after 8 weeks.



EXPECTED RESULTS

6. Target locus analysis would bear out the nuclease efficacy at the target site and higher rate of NHEJ expected from TALEN delivered as DNA species.

9. Screening of subpool cells would be positive for PCR product (minimum 80%). Moreover, subsequent clones would show higher frequency of correction when using DNA as a delivery vehicle.

11. TALEN-modified cells with the corrected mutation would recover protein production.

12. GFP IDLV insertion would be showed only when TALENs are cointroduced with the IDLV. Furthermore, after the IDLV delivery we would see a rapid loss of GFP. In contrast, the cointroduction of IDLV and TALENs would result in a stable population of GFP cells. *In silico* studies show that our TALEN has no off-target sites, our study would confirm this data.

14. The engraftment of human CD45$^+$ cells would be comparable with mice injected with manipulated and UM cells in all the organs tested. No significant differences would be observed between different treatment groups neither in terms of level engraftment nor in differentiation pattern in tested organs. Therefore, corrected cells would be present in all hematopoietic organs.

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