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Gene Editing as an Alternative to Retroviral Vectors for Wiskott-Aldrich syndrome Gene therapy.

Edición génica como alternativa a los vectores retrovirales para terapia génica del síndrome de Wiskott-Aldrich

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The best scientist is open to experience and begins with romance - the idea that anything is possible.

Ray Bradbury

A mis padres

La doctorando M. Alejandra Gutierrez Guerrero, el tutor Francisco Martín Molina y los directores de Tesis Francisco Martín Molina y Karim Benabdel Lah El Khlanji, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección del tutor y directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo se han respetado los derechos de otros autores a ser citados cuando se han utilizado sus resultados o publicaciones.

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“Develop an attitude of gratitude, and give thanks for everything that happens to you, knowing that every step forward is a step toward achieving something bigger and better than your current situation.”

Brian Tracy

Después de 4 años, me enfrento a mi última página en blanco, seguramente la más importante, la que, aunque no lo parezca, es la más difícil y la que todo el mundo busca cuando abre la tesis. Aguantad los cambios a los diferentes idiomas, por favor, Gracias!

After 4 years, I face my last blank page, definitely the most important, the one that although it doesn't seem like it, is the most difficult and the one that everyone turns to when opening the thesis. Bear with me the language changes, please, Thank you

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“The most exciting phrase to hear in science, the one that heralds new discoveries, is not ‘Eureka!' but 'That's funny...' ”

Isaac Asimov

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RESUMEN / ABSTRACT

“ Science is not only a disciple of reason but, also, one of romance and passion"

Stephen Hawking

Resumen

Uno de los tratamientos más eficientes y seguros para enfermedades monogénicas que afectan al sistema hematopoyético es la terapia génica mediante vectores lentivirales (LVs) y gammaretrovirales (GVs). Sin embargo, a pesar de los claros beneficios terapéuticos obtenidos con las últimas generaciones de estos vectores, ambos son vectores retrovirales que se integran al azar en sitios activos de transcripción. Esta integración incontrolada genera un riesgo de mutagénesis insercional que puede generar efectos indeseados en las células modificadas. La edición génica se ha propuesto como una alternativa más segura, cuyo objetivo es la restauración de niveles terapéuticos del gen endógeno o bien la inserción ectópica del gen terapéutico específicamente en el mismo locus mutado o en un locus seguro (*safe harbour*). Las estrategias de edición génica más potentes se basan en la utilización de nucleasas específicas que producen roturas de doble cadena (DSB) en un sitio específico del genoma. Los procesos de reparación mediante unión de extremos no homólogos (NHEJ) o mediante recombinación homóloga (HR) permiten editar el genoma de diferentes maneras para logar un mismo objetivo final: recuperar la función normal del gen mutado. Nuestro objetivo final es desarrollar unas herramientas de edición génica que sean eficientes para el rescate de células humanas hematopoyéticas (hHSC) para pacientes que presentan el síndrome de Wiskott-Aldrich (WAS).

En este trabajo, hemos diseñado diferentes sistemas de CRISPR/Cas9 específicos para WAS y las hemos comparado frente a las ZFNs (*zinc finger nucleases*) homodiméricas y heterodiméricas dirigidas exactamente a la misma región utilizando nucleofección y lentivirus deficientes para la integrasa (IDLVs) para su entrega en K562. Nuestros resultados muestran que, aunque el sistema CRISPR/Cas9 puede ser tan eficiente como la mejor ZFNs en cuanto a disrupción génica (reparación mediante NHEJ), el resultado depende del sistema de entrega. De hecho, mientras que los dos sistemas tienen una eficiencia similar mediante nucleofección, la entrega con IDLVs de las ZFNs consigue eficacias superiores de corte en el locus WAS que la entrega de del sistema CRISPR/Cas9. Esto es cierto incluso para los nuevos sistemas CRISPR optimizados mediante la co-expresión del RNA guía (gRNA) y Cas9 en el mismo vector (sistemas *all-in-one*), la inclusión del elemento WPRE (del inglés *woodchuck hepatitis virus post-transcriptional regulatory element*) o la inserción de aisladores. Nuestros estudios también demostraron que nuestro sistema CRISPR/Cas9 tenía una especificidad similar a las ZFNs heterodiméricas y superior a las ZFNs homodiméricas. Estos resultados fueron obtenidos gracias al análisis de los niveles de DSBs detectados (tinción con γH2AX) en células editadas con las diferentes nucleasas. Interesantemente, cuando se analizó la eficiencia y especificidad de los diferentes sistemas para la edición génica del locus *WAS* mediante HR (inserción de un *cassette* de expresión en el locus *WAS*), pudimos observar un patrón parecido a lo observado por NHEJ: Las ZFNs heterodiméricas y el sistema CRISPR/Cas9 muestran una especificidad similar, consiguiendo alrededor de un 80% de integraciones del ADN donador en el locus *WAS* (*in target*). Sin embargo, las ZFNs homodiméricas muestran una baja especificidad, mostrando alrededor de un 55% de inserción del donador fuera del sitio diana. Datos que confirman los resultados obtenidos con el análisis de especificidad utilizando la tinción de γH2AX.

Todos los primeros análisis fueron realizados en las células K562 con la finalidad de su optimización y comparación en un modelo celular más fácil de trabajar. Sin embargo las células diana para terapia génica de WAS son las células madre hematopoyéticas (HSCs) y/o las células T. Por lo tanto procedimos a investigar la eficacia de los sistemas ZFNs y CRISPR/Cas9 para editar WAS en estos tipos celulares. Sin embargo, ninguno de estos sistemas logró niveles detectables de corte del locus *WAS* en HSCs o células T. Se procedió por tanto, en la última parte de esta tesis, a investigar otro sistema de entrega del sistema CRISPR. En colaboración con los grupos de Els Verhoeyen y Theophile Ohlmann se desarrollaron VLPs (del inglés *virus like particles) o nanoblades* dirigidos frente al locus *WAS*. Se produjeron *nanoblades* derivados

de MLV y HIV-1, incorporando Cas9 y gRNAs y pseudotipados con diferentes envueltas. Las WAS-*nanoblades* basados en MLV e incorporando simultáneamente las envueltas de retrovirus de babuino modificada (BRL) y VSVg fueron las que presentaron una mejor eficacia, siendo capaces de editar alrededor del 30% del locus *WAS* tanto en células HSC como en células T. Queda por investigar si las *nanoblades*-ZFNs pueden mejorar la eficiencia/especificidad de edición de las *nanoblades*-CRISPR/Cas9. En cualquier caso, las *nanoblades* CRISPR/Cas9 han demostrado ser un sistema novedoso y potente para su futuro uso terapéutico debido a su alta eficiencia para editar el genoma de células primarias como las HSCs y las células T.

Abstract

One of the safest and most efficient treatments for monogenic diseases affecting the hematopoietic system is gene therapy using lentiviral vectors (LVs) and gammaretroviral vectors (GVs). However, although latest generation LVs and GVs are highly efficient and safe, both are retroviral vectors which integrate randomly in transcriptional active sites. This uncontrollable integration generates risk of insertional mutagenesis, which can produce undesirable effects on the modified cells. Gene editing has been proposed as a safer alternative, whose objective is the restoration of the endogenous levels of the wild type gene or the ectopic insertion of a therapeutic gene in the mutated locus or in a safe locus (safe harbor). The most potent gene editing strategies are based on the use of specific nucleases that produce double strand brakes (DSBs) in a specific site in the genome. Non homologous end joining (NHEJ) or homologous recombination (HR) repair of these DSBs allow editing the genome in different ways with a final objective: restore de normal function of the mutated gene. Our final aim was to develop a gene edition tool for efficient genetic rescue of human hematopoietic stem cells (hHSCs) from Wiskott-Aldrich syndrome (WAS) patients.

In this work, we have designed different *WAS*-specific CRISPR/Cas9 systems and compared their efficacy and specificity with homodimeric and heterodimeric *WAS*-specific ZFNs (zinc finger nucleases) directed to exactly the same region, using nucleofection and integrative-deficient lentiviral vectors (IDLVs) as delivery systems in K562 cells. Our results showed that, although the CRISPR/Cas9 system can be as efficient as the best ZFNs for gene disruption (by NHEJ); the outcome depends on the delivery system. Indeed, while both systems achieved similar results using nucleofection, IDLV delivery of ZFNs outperformed the IDLV delivering CRISPR/Cas9 system. This is true even for our optimized CRISPR system by the co-expression of the guide RNA (gRNA) and Cas9 in the same vector (all-in-one system), the inclusion of the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) element or the insertion of insulators. Our studies also showed that our *WAS*-specific CRISPR/Cas9 system has a similar specificity to the *WAS*-specific heterodimeric ZFNs and is higher to the *WAS*-specific homodimeric ZFNs. These results were obtained thanks to the analysis of DSBs levels detected (γH2AX staining) in the edited cells with the different nucleases. Interestingly, when we analyzed the efficacy and specificity of the different systems for genome edition of the *WAS* locus by HR (insertion of an expression cassette in the *WAS* locus), we observed a similar pattern to that obtained with NHEJ; heterodimeric ZFNs and CRISRP/Cas9 system show a similar specificity, achieving around a 80% of donor DNA integration in the *WAS* locus (in target). However, homodimeric ZFNs show a low specificity, around a 55% of donor insertion outside the target site. Data that confirmed the results obtain by the analysis of specificity by γH2AX staining.

All the first analyses were done in K562 cells with the purpose of optimization and comparison in an easier cellular model. However, the target cells for WAS gene therapy are hematopoietic stem cells (HSCs) and/or T cells. We decided to study the efficiency on the ZFNs and CRISPR/Cas9 system to edit *WAS* in these cells type. However, none of these system were able to achieve the desirable levels of cutting in the *WAS* locus in HSC or T cells. In the last part of this thesis, we decided to study other delivery systems for CRISPR. In collaboration with Els Verhoeyen group and Theophile Ohmann group, VLPs (virus like particles) or nanoblades were developed against the *WAS* locus. Nanoblades derived from MLV and HIV-1 were produced, incorporating Cas9 and gRNA and pseudotyped with different envelops. The *WAS*-nanoblades based on the MLV pseudotyped with the modified Baboon endogenous retroviral (BRL) and VSVg envelopes were the ones that presented the best efficiency, being able to edit around a 30% of the *WAS* locus both in HSC cells and T cells. There is still need to investigate if the ZFNs-nanoblades could improve the efficiency and specificity of the CRISPR/Cas9-nanoblades. Nevertheless, the CRISPR/Cas9 nanoblades have shown to be a new and strong system for future therapeutic use, due to their high efficiency in editing the genome in primary cells such as HSC and T cells.

INTRODUCTION

"There is nothing more difficult to take in hand, more perilous to conduct, or more uncertain in its success, than to take the lead in the introduction of a new order of things."

Niccolo Machiavelli

Introduction

1. Wiskott Aldrich Syndrome

Frederic Wiskott, in 1937, described that the symptoms suffered by three brothers whom he was studying, but not their sisters, presented a novel hereditary thrombopathy. In 1954, Robert Aldrich reported a similar phenotype he had studied for over six generations in a family, where only some of the males and none of the females showed this characteristic phenotype, concluding that it was an X-linked inherited disease. As a result this novel disease was named Wiskott-Aldrich syndrome (WAS) after the name of both physicians [1]. It was only later, that different attempts were made to identify the region where the *WAS* gene was located. Finally, Derry *et al.* identified the gene by positional cloning on the X chromosome (position Xp11.22-p11.23) [2].

Today, WAS has been described as a recessive, severe, primary immunodeficiency linked to the X chromosome. The *WAS* gene consists of 12 exons resulting in a cDNA of 1823 base pairs, which encodes a 502 amino acid protein, the WAS protein (WASP), and has a molecular weight of 54kDa, exclusively expressed in hematopoietic cells [3, 4]. Importantly, a strong correlation exists between the severity of WAS and the levels of WASP.

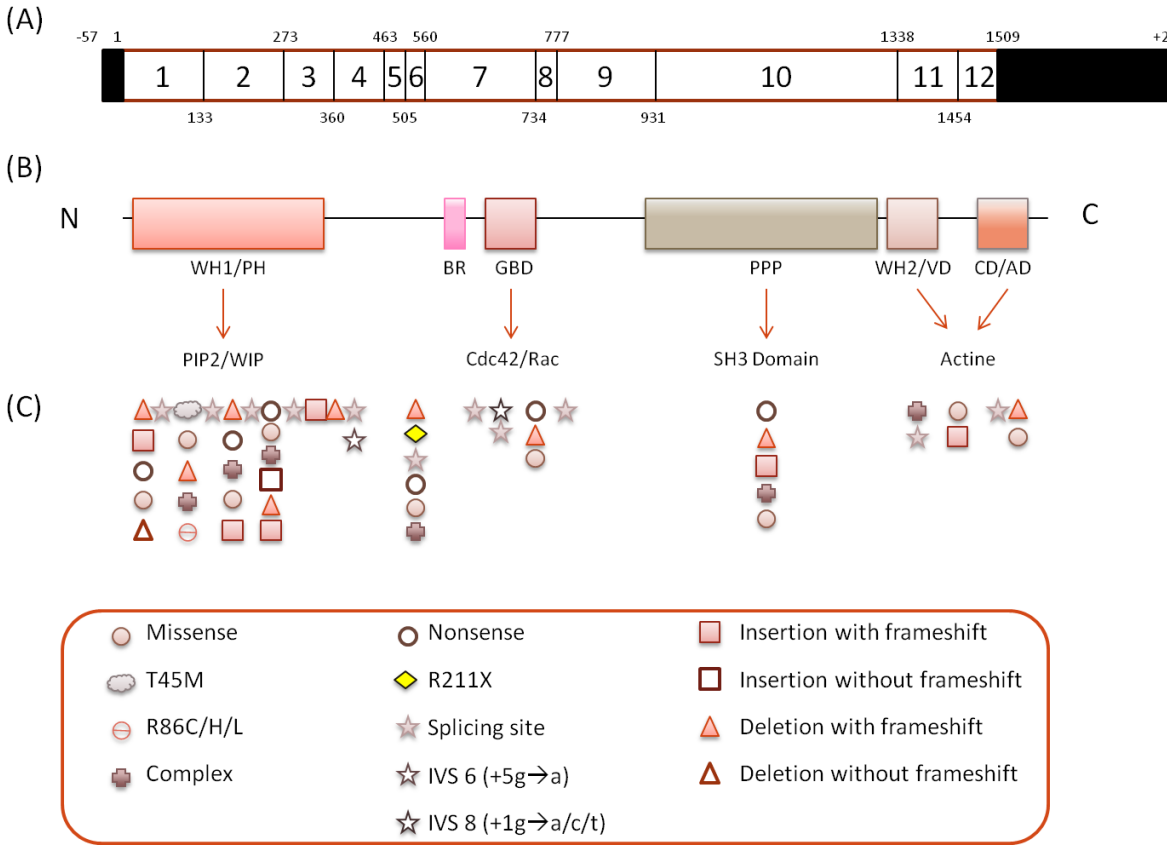


Figure 1. Schematic illustration of WAS representing the 12 exons and the major functional domains. A) Schematic representation of *WAS* gene organization and protein domains. The 12 exons of the *WAS* gene are indicated. The numbers above and below the *WAS* gene indicate the starting nucleotide number of each exon. The untranslated regions of the first and last exon are shown in black. **B)** Schematic representation of WASP, showing the major functional domains: WH, WASP homology; PH, pleckstrin homology; BR, basic region; GBD, GTPase-Binding domain; PPP, proline rich region; VD, verprolin domain; CD, cofilin domain; AD, acidic domain. **C)** Distribution of known mutations of the *WAS* gene; each symbol represents each type of mutations (symbol legend in the box). Figure modified from [1, 3, 4].

The worst case scenario is when WASP is totally absent, leading to defects in the multiple hematopoietic lineages. There is the lymphoid linage which will give B cells, natural killer cells (NK) and the different types of T cells and then there is the myeloid linage that will give all the blood cell types; erythrocytes, neutrophils, basophils and eosinophils as well as megakaryocytes, platelets, macrophages and dendritic cells. On the other hand the decrease in WASP levels has different consequences for the different blood cell types, being platelets the most affected [5, 6].

According to the Resource of Asian primary immunodeficiency diseases there have been around 300 unique mutations spread along the *WAS* gene. Being the most frequent the missense mutations, followed by splice-site mutations, deletions, nonsense mutations, insertions and complex mutations [1, 7, 8]. The locations of the most abundant mutations are summarised in Figure 1.

The clinical scoring system is derived from a variety of clinical parameters, including the presence of thrombocytopenia, eczema, immunodeficiency, autoimmunity, and malignancy. The WAS scoring system ranges between 0 and 5. Patients with small amounts of WASP are scored 1 or 2 and suffer from X-linked thrombocytopenia (XLT), which is characterized by microthrombocytopenia but no other immunological defects (score 1) or platelet defects, mild eczema and infections (score2).

When thrombocytopenia and small platelets are present and there is a persistent eczema and patients suffer from recurrent infections they are considered WAS patients and they get attributed a score 3. When the eczema is difficult to treat and they have multiple infections, they belong to a score 4. Finally, a score 5 is attributed when in addition to having persisting eczema and/or frequent infections, patients also develop autoimmune diseases and other malignancies, such as lymphoma [1, 7-9].

The main symptoms of this disease are eczema, thrombocytopenia and recurrent infections. Only some patients can show multiple tumour formations (lymphoma) and autoimmune diseases, which reduce their life span drastically [1]. It is believed that the development of these malignancies could be due to the decrease on the cytotoxic T cells, which normally kill cancer cells [10] as there is a decrease in lymphocyte counts as well as defects in T and B cell function. It has been reported that the WAS T cell is biased towards the TCRVβ repertoire. Although it is suggested that this is associated to WASP deficiency, it is not related with normal TCR formation in the thymus. It is thought that it is with time that the oligoclonality appears, caused by the absence of WASP [11].WAS patients that have a low number of immature B cells and memory B cells proliferate slowly. These cells are not able to completely activate as they have a disruption in the CD19-Btk signalling. This might be explained through the effect of WASP on CD19 transcription and because of its absence, B cells response is defective [12].WAS patients also exhibit variable levels of Immunoglobulin E (IgE), which normally is associated with allergic diseases [13] and could explain the imbalance of Th1/Th2 cytokines causing the eczema [14, 15].

Other clinical phenotypes are also frequently detected, such as haemorrhages caused by thrombocytopenia with small platelets, epistaxis, petechia, gastrointestinal haemorrhages and intracranial bleeding. The most common diseases related to autoimmunity are haemolytic autoimmune anaemia, arthritis, nephropathy and coetaneous vasculitis [16]. It is common for WAS patients to develop more than one of these diseases at the same time.
All these autoimmune events can lead to tumour development. The incidence rate of tumour development is 22% during childhood (especially myelodysplasia), however it is more frequent in teenagers and young adults, being the most common leukaemia, myelodysplasia and lymphoma, representing 25% of deaths in WAS patients [16].
Even though there have been lots of advances in diagnosis and clinical care, the survival prognosis for WAS patients is still low. The first step is normally to treat the symptoms. For example, splenectomy to increase the levels of platelets to normal levels; however, this does not reduce the risks of autoimmunity and the appearance of lympho-proliferative disorders [17, 18]. Normally, the function of B and T cells is reduced in splenectomised patients who get a hematopoietic stem cell transplantation (HSCT) [19], which is the treatment of choice for WAS patients.

The HSCT can correct all aspects of the disease, providing hematologic and immune reconstitution [20]. Patients need conditioning with busulfan and immune suppression with cyclophosphamide to ensure high

level donor chimerism [21]. To achieve the best outcome it is preferable if the donor is an identical HLA sibling donor or matched un-related donors since mismatched un-related donor transplanted patients show lower survival rates when the patients are younger than 5 years old [19]. However, there has been great advances in patient conditioning and survival of patients older than 5 years old when using a matched un-related donor or using matched cord blood as a stem cell source [22].

2. Gene therapy

a. General facts

Gene therapy can be defined as the introduction of therapeutic genes into target-cells, to treat a medical disorder or disease. This is especially relevant in the treatment of monogenic diseases, in which the genetic defect can be corrected by a therapeutic gene [23, 24].

Genetic material delivered into target cells can be DNA or RNA and they can be used with the aim of repairing a genetic defect or to confer a novel function. In this context, the therapeutic transgene can have different functions: to replace the function of a mutated gene, to increase the production of a physiologic substance, or to produce a substance not present in the target organism [25] .

There are three main players in any gene therapy strategy: the target cells, the transfer vector and the therapeutic transgene:

- **The target cells** or tissue represent the selected cell type for gene modification. They can be cells affected by the disease or not. The cell type which can be used for therapeutic gene modification depends on the disease. They can be fibroblasts, neurons, hepatocytes, hematopoietic stem cells (HSC), mesenchymal stem cells (MSC), B and T cells, among others [25].

- Gene Transfer vectors are systems that allow the introduction of genetic material into target cells. Gene transfer vectors need to satisfy 3 main criteria; they should protect the transgene against degradation, bring the transgene across the cell membrane, and they should have no damaging effects. They are divided into two categories; non-viral and viral. The latest are the most highly developed and exploited for gene therapy [26] .

The main challenge of gene therapy is to get a large percentage of target cells expressing the therapeutic gene without affecting their normal physiology. In this direction viral vectors have the best profile for almost all diseases.

During the 1960s and early 1970s the concepts of gene therapy appeared next to the development of labelled cells and the elucidation of the mechanisms of cell transformation by different viruses, such as papovaviridae (Simian vacuolating virus 40) and polyomavirus. Recombinant DNA techniques became available to demonstrate that foreign genes could perfectly correct genetic defects improving disease phenotypes, at least *in vitro* [27].

It was during these decades that the first gene therapy trial took place. The Shope papilloma virus was inoculated intravenously to treat hyperagininemia, the virus was encoding arginase. The vector was previously tested in rabbits where the arginine blood levels were normal and no secondary effects were seen. However, arginine blood levels in human patients didn't decrease as it was observed in rabbits, failure of the therapy was attributed to the inactivation of the virus *in vivo* [28, 29]. By the 1980s, gene transfer was performed commonly in mammalian cells. Actually, it was seen that retrovirus-based gene transfer presented advantages for the treatment of inherited diseases, because of the integration of foreign material in the host cell genome. However, it was highly controversial due to the potential influences that the integrated foreign DNA could have in the host cells.

In 1995, the first results of a clinical trial using gene corrected HSCs to correct adenosine deaminase deficiency (ADA) was published by Donald B. Kohn *et al* [30]. Conversely, the expression of the therapeutic transgene was transient, which led to the inability to abandon ADA replacement treatment. Importantly, this showed that if the efficiency could be increased it was possible to use gene therapy safely and therapeutically. Five years later, a new publication finally reported a successful treatment for children

suffering from X-SCID in France. The scientists used a gamma-retroviral vector (based on murine leukaemia virus-MLV) to express the common interleukin receptor γ-chain (the protein lacking in the SCID-X1 children). After 9 years, 8 of the 9 patients treated on the trial were alive and had normal lives. This successful treatment was overshadowed 2 years and a half after treatment when one of the patients developed a T cell leukaemia due to insertional mutagenesis [31]. Patients from different trials went on to develop similar leukaemia, although in those cases they were treated with chemotherapy successfully and are in complete remission [32].

An alternative therapeutic strategy using gene modified HSCs consists in using them as Trojan horses to deliver the therapeutic protein to target tissues. However, in most of these cases, the over-expression of the transgene does not render selective advantage to the HSCs and is therefore necessary to increase efficiency of the retroviral vectors. The first successful report using this strategy targeted the X-linked adrenoleukodystrophy (X-ALD). The authors transplanted patients with autologous HSCs transduced with lentiviral vectors (LVs) expressing ALD. Since the gene modified cells did not show a selective advantage, patients were conditioned to favour engraftment of gene-modified cells [33]. Even though it has been reported that the two-treated patients expressed the X-ALD protein in multiple lineages, it is only effective if gene therapy is performed in early stages of the disease, and on top of that larger cohorts of patients are needed to evaluate the efficacy. Nevertheless, this was the first report of LVs being successful in treating, not only a genetic disease but a severe brain disease too. A step forward in terms of efficacy using a similar strategy was observed in a clinical trial in Milan (Italy) targeting metachromatic leukodystrophy (MLD). The authors showed complete blockage of disease progression in children that did not have any alternative treatment and an average life expectancy of 4 years [34, 35].

The principal investigators of the SCID-X1 and SCID-ADA trials agreed that the success of gene therapy was due to the selective advantage of the genetic modified cells in comparison to the defective cells [36, 37]. At the moment, there are more than 2000 clinical trials in gene therapy completed, ongoing or approved worldwide which try to evaluate the benefits and problems derived from its application (http://www.abedia.com/wiley/continents.php).

b. Gene therapy for Wiskott-Aldrich syndrome

Gene therapy can be considered as the treatment of choice in WAS patients with a high disease score, who do not express the protein and have no compatible HLA donor for transplantation. The therapy is based on the isolation of $CD34^+$ cells from the patient, which are transduced *ex vivo* with a viral vector that contains the *WAS* transgene. Then these cells expressing the *WAS* transgene are introduced into the patient [38].

The first *WAS* gene therapy trial was performed in two patients using a GV containing the WAS transgene with a 3 year follow up [39]. The patients continued to express WASP in $CD34^+$, lymphoid, myeloid cells and platelets after 3 years post-gene therapy, meaning that T, B and NK cells were able to recover their function. However, although after the first year of the transplant with the modified cells, all symptoms of autoimmunity had disappeared, the severe eczema only improved in one of the patients [40] and seven of them developed leukaemia, probably due to insertional mutagenesis [41]. It was shown that all patients who exhibit leukaemia had a retroviral insertion close or in the *LMO2* gene, suggesting that high levels of LMO2 are triggering the leukaemia [38, 42]. Due to these facts, the choice of the viral vector was reconsidered and the next trials were performed with the safer self-inactivated (SIN) LV.

The latest clinical trial for WAS using gene therapy on 3 patients with SIN-LV includes the 1,6kb-*WAS* proximal promoter. After more than 20 months upon transplantation of corrected HSCs, all 3 patients resolved their eczema as well as the intestinal bleeding and the epistaxis [43]. Up to date there are 5 WAS gene therapy clinical trials using LVs; one in Italy, one in Boston (USA) and three in France [44, 45].

3. Gene editing

Gene editing is a type of genetic engineering where the DNA is either inserted, deleted or replaced in the genome using specific engineered nucleases, which create a site-specific double-strand break (DSB). The cell machinery repairs this DSB either by non-homologous end joining (NHEJ) or by homologous recombination (HR) when a DNA template with homology sequences is given (Figure 2). The advantages of gene editing over gene addition are 1) the capacity of modifying specific endogenous sequences, 2) the

transcriptional regulation of the gene is maintained since correction can be introduced at the endogeneous site and thus will be under the control of the natural promoter and 3) the insertional mutagenesis risks and the activation of oncogenes are reduced [46].

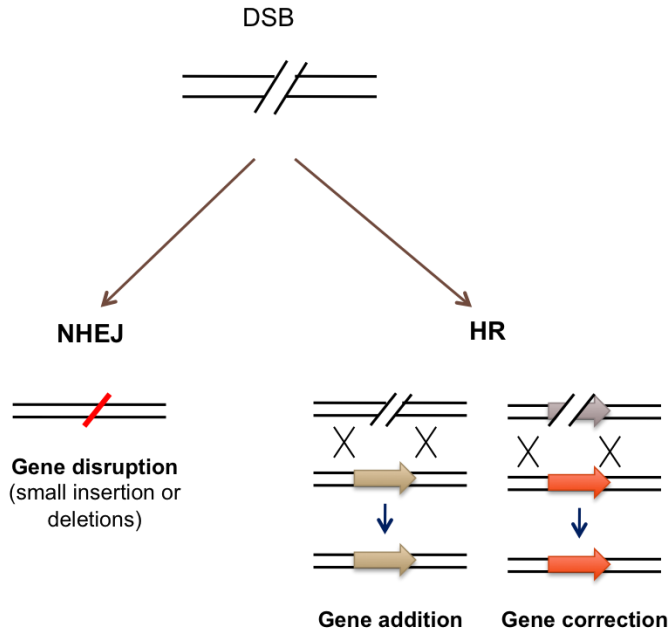


Figure 2. DNA double-strand break (DSB) repair. The DSB made by the specific endonucleases are repaired by the cell mechanisms, predominantly by two pathways: Non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ pathway creates small deletions or insertions creating a disruption in the gene (scheme on the left). However, HR uses homologous template, usually a sister chromatid under natural circumstance, or artificially delivered homologous DNA (scheme on the right). This approach allows the generation of specific mutation.

a. Nucleases

Programmable nucleases generate DBS in a specific locus in the genome. There are four different types of nucleases, which have been successfully engineered for biotechnology: Meganucleases (MGNs), transcription activator-like effector nucleases (TALENs), zinc fingers nucleases (ZFNs) and clustered regularly interspaced short palindromic repeats associated to Cas9 nucleases (CRISPR/Cas9) system. They are designed to produce these DSBs in a specific locus. However, they can also produce them in other parts of the genome, what its call off-targets. The off-target activity is linked to areas of the genome which share high homology with the target site [47] .

i. Meganucleases

MGNs are endonucleases that recognise 12-45bp sequences of DNA and they make the cut in the homology arm [48-50] (Figure 3A). Their main limitation is the available repertoire. Although there has been a screening of all the MGNs, they only cover a small number of targets. To solve this problem MGNs are engineered and fused with other domains to create chimeric proteins [51]. However, this is a time-consuming procedure and, most importantly, only a small number of target-specific MGNs have been developed.

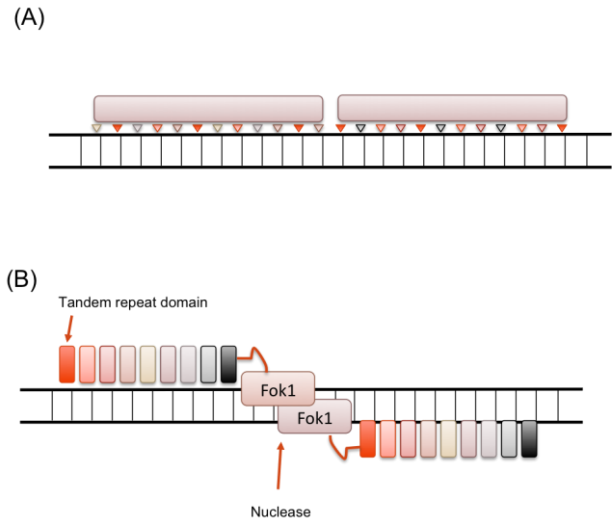


Figure 3. **A)** Meganucleases. The MGNs possess a DNA recognition and cleavage in a single domain. **B)** TALENs. The DNA binding domains are formed by tandem high repeated sequences, which recognize each nucleotide, and the catalytic domain is formed by the Fok1 endonucleases. It's formed by two arms (left and right) with an endonuclease each. Modified from [52].

ii. Transcription activator-like effector nucleases (TALENs)

The transcription activator like effector (TALE) protein is secreted by bacteria of the *Xanthomonas* family [53]. It has been reported that this protein possess homologous domains of about 33-35 amino acids repeated in tandem for 10-30 times. The specificity of each repeat is given by the repeat-variable di-residues in position 12 and 13 of each repeat, conferring specificity of each repeat to a single nucleotide on the target sequence [54, 55]. The TALE nuclease (TALEN) is a combination of a N-terminus domain that includes a nuclear localization signal (NLS); a central domain where the tandem repetitions of the TALE are included and a C-terminus domain where a Fok1 nuclease is placed [56] (figure 3B). The TALEN is only active as a dimer so two TALENs must be designed to bind to the target locus in a face to face fashion to achieve cleavage of the target sequence [57]. Different TALENs have been generated since 2009 when the interaction TALE-DNA was discovered due to their relatively easy design [58, 59]. However, their size (more than 3kb) and highly repeated nature prevent them from easily packing into viral vectors for their delivery.

iii. Zinc Finger nucleases (ZFNs).

Zinc finger nucleases are artificial domains engineered by 3-5 Cys_2-Hys_2 zinc finger domains fused to the catalytic domain of the Fok1, a type II S bacteria endonuclease [60]. Each zinc finger domain binds specifically to DNA by the recognition of nucleotide triplets; the combination of different zinc finger domains recognizes a longer DNA sequence that depends on the number of zinc fingers. (Figure 4A) As for TALENs, the cleavage activity of ZFNs needs dimerization (since Fok1 is active as a dimer).

Therefore two ZFNs must be designed; each ZFN must target a DNA sequence that is separated by a short sequence from the recognition site of the other ZFNs in a head to head fashion (Figure 4A). Their off-target activity has also been reported [61, 62] however, they have been almost overcome by mixing to different Fok1 domains that are forced to form heterodimers. In these optimized ZFNs, the only way to create a DBS is when the two different ZFNs bind to its specific target DNA site [63, 64] (Figure 4B). Even though there are some open-source libraries for their design [65, 66], the engineering remains difficult.

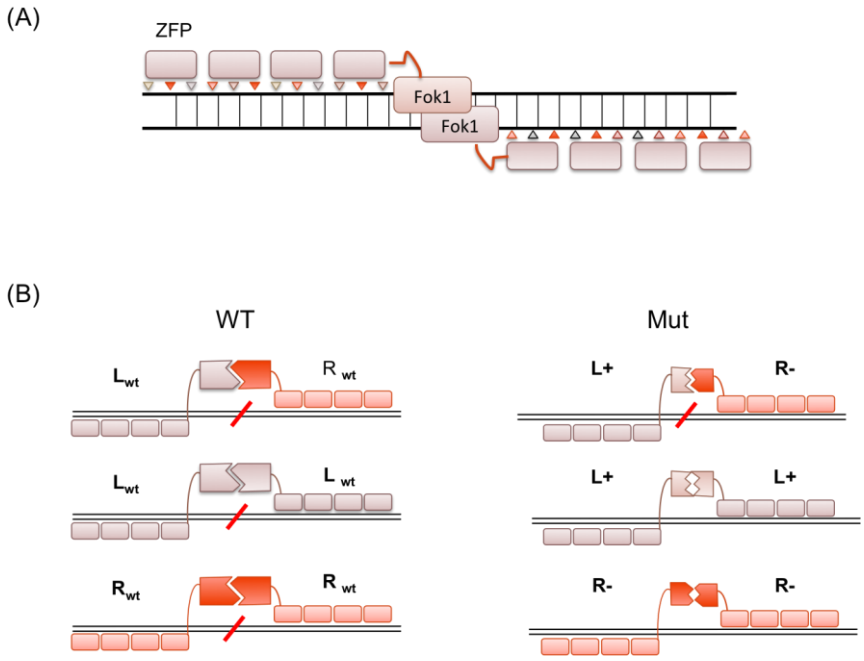


Figure 4. Zinc finger nucleases **A)** Scheme of the ZFNs. They are formed by two subunits, the biding domains which form homology by groups of 3 nucleotides and the cleavage domain formed by the Fok1 nuclease. **B)** Scheme of the two arms that form the ZFNs (Left-L and Right-R) with the Fok1 endonuclease (polygons). Left scheme shows the wt Fok1 (self-complementary polygons) which produce cleavage (red inclined line) in all the different combinations (heterodimer (top) and homodimers (middle and bottom). Right scheme shows the modification of the Fok1 endonucleases (complementary polygons) and their different combinations, where only when two different arms (heterodimers) are present the cleavage (red inclined line) appears and when the arms are the same (homodimer) no cleavage occurs (middle and bottom). Modified from [63].

iv. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

The CRISPR/Cas9 system is a new, easy to design and highly efficient gene editing tool. It is derived from the adaptive immune system found in prokaryotes, which provides a defence mechanism to some viral infections and plasmids.

There have been three CRISPR/Cas systems identified (I-III) in *archae* and bacteria; with *cas3, cas9* and *cas10* being the signature gene for type I, type II and type III systems respectively. The type II is the most versatile for genome engineering purposes. This type II system encodes the Cas9 protein that has two domains with nuclease activity (type RuvC domain in the N-terminus and a type HNH domain in the middle of the protein). This protein forms a complex with two other RNA molecules: crRNA (CRISPR RNA) and tracrRNA (crRNA transactivator).

For the CRISPR/Cas9 system, the transcripts from the CRISPR repeat arrays are processed into RNA (crRNA), each containing sequences from exogenous DNA. It is formed by 42 nucleotides, 20 of them are the protospacer sequence and the other 22 are the repeated sequence. The tracrRNA, which complements the crRNA, is needed for the crRNA maturation and for the cleavage by the Cas9. Together, the crRNA and the tracrRNA form a double RNA strain that guides the Cas9 to the target DNA and produces the cleavage [67]. If they are adjacent to a short sequence known as the protospacer adjacent motifs (PAMs), NGG for Cas9 from *Streptococcus pyrogenes*, the Cas9 creates a 3bp DSB upstream of the PAM (Figure 5). The RNA components can be combined into a single guide RNA (gRNA)[68]. The only part that needs to be changed to specifically cut a new target site in the genome is the 20 nucleotides in the crRNA and the PAM motif in the genome [68, 69]. It has been reported that the off-target activity of some CRISPR/Cas9/gRNA systems can be higher than other specific nucleases [70]. Different modifications to the gRNA design and to the Cas9 have been performed to reduce off target activity. Some of these modifications are; 1) the reduction of the gRNA protospacer length, which makes the gRNA more tolerant

to mismatches and less likely to bind to off-target sites [71]; 2) the generation of mutated Cas9 [72], where the aspartate in position 10 is substituted by alanine (D10A) in the RuvC domain making it impossible for the Cas9 to cut the 5'→3' strain acting as a nickase, in this case two gRNA have to be designed.

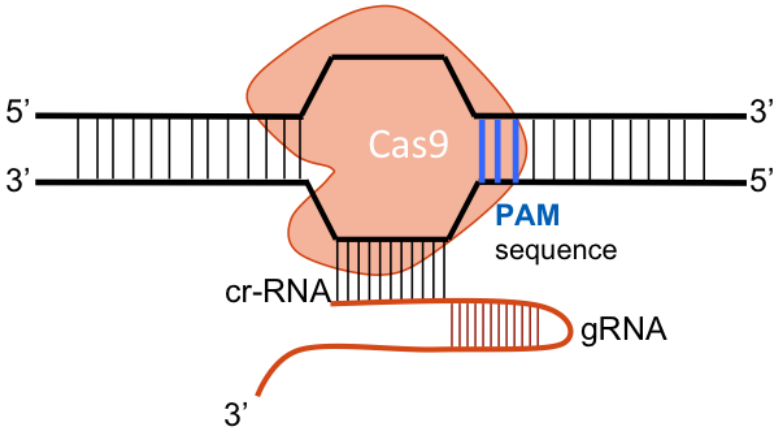


Figure 5. CRISPR/Cas9. Cas9 has the cleavage activity and a gRNA gives the specific region to bind into the genome and directs the Cas9. Modified from [52].

4. Delivery of Nucleases and donor DNA.

In order to efficiently edit any cell type, a suitable delivery system is needed to get the endonucleases and/or the donor DNA into the nucleus of the target cells. Both *ex vivo* and *in vivo* genome editing can be achieved having their advantages and drawbacks. However, *ex vivo* genome editing have more probabilities to get into the clinic since higher editing rates can be achieved and the medicinal product (genome edited cells) can be monitored for potential alterations before transplantation [73]. Both, viral and non-viral gene delivery systems have been used successfully for genome edition of different target cells. However, for several applications, the main drawback is the manipulation and culture of the target cells outside the body, as they are prone to lose their properties and/or their ability to engraft. *In vivo* therapy is able to target a wide variety of cell populations, allowing treatment of multiple organs and diseases that wouldn't be possible with the *ex vivo* approach. For *in vivo,* viral vectors are the vectors of choice due to their higher efficacy compared to non-viral systems. However, depending on the viral system used they are limited by the package capacity (Adeno associated virus), and the immune response (adeno associated virus, adenovirus), as well as for their production costs (adeno associated virus, adenovirus and integrative deficient LVs (IDLVs)). This is why non-viral systems are taken into consideration even for *in vivo* applications [74, 75].

In the case of the donor DNA the most common delivery systems are nucleofection of DNA as a plasmid, single stranded DNA (ssDNA), IDLVs and AVV. Depending on the method of the delivery, the strategy to transfer the different compounds might change.

Viral vector systems can deliver both, the nucleases and the donor DNA in the same vector or as different vector particles. Viral vectors are derived from viruses and deliver genetic information into cells for gene therapy purposes. They are normally effective for gene editing in immortalized cells, but less in primary cells, nevertheless they are normally more efficient than non-viral methods for primary cells. Figure 6 shows the percentage of the different delivery strategies used for gene editing studies.

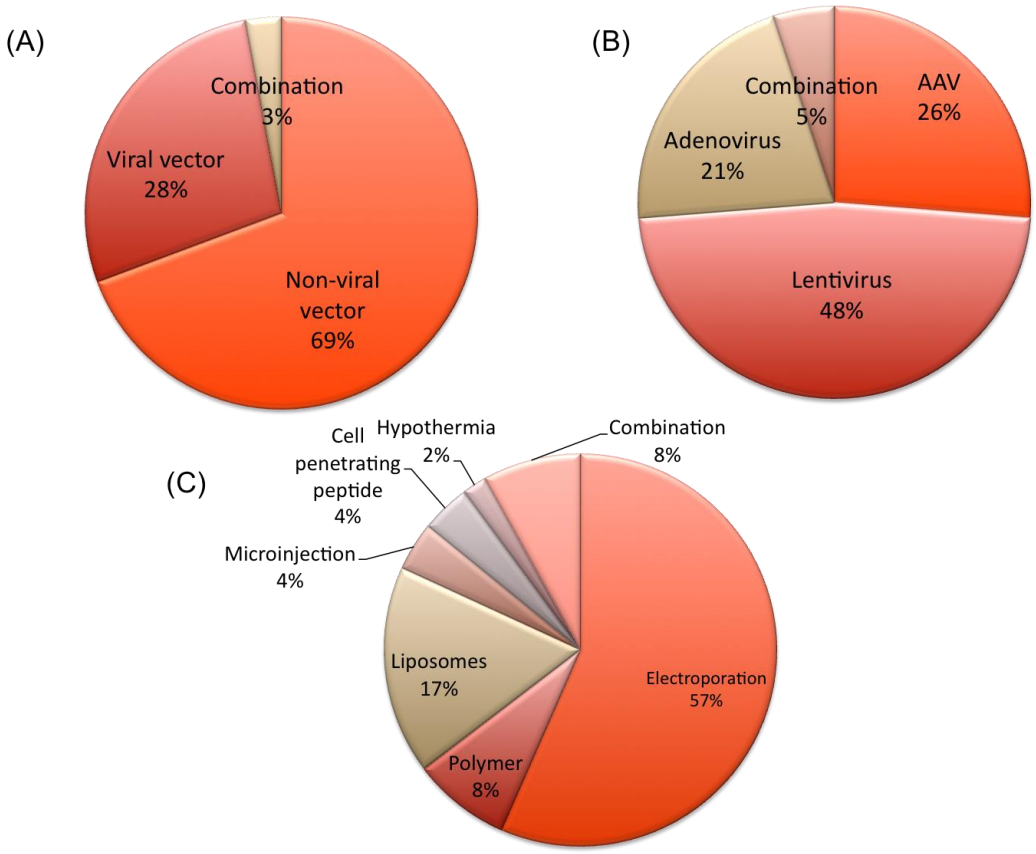


Figure 6. Delivery strategies for gene editing studies. Percentages of therapeutic gene editing of non-clinical development based on **A)** delivery systems, **B)** type of viral vector delivery and **C)** type of non-viral delivery. Modified from [52].

a. Non-Viral

Non-viral vectors systems have two components: the material to be delivered (DNA, ssDNA, proteins or RNA) and the transfer system. To deliver the nuclease it is possible to use DNA or RNA coding for the nuclease or the nuclease as a protein itself. To deliver the donor DNA, Plasmids or ssDNA are used. The material is based on plasmids, which consist of circular DNA, linear ssDNA or RNA. The different delivery systems can be physical, such as carrier-free gene delivery or chemical by synthetic vector-based gene delivery. The physical approach uses physical force to penetrate the cell membrane and facilitates intracellular gene transfer. It includes electroporation, needle injection, gene gun, ultrasound and hydrodynamic delivery. On the other hand the chemical approach uses synthetic or natural compounds as carriers [76].

These types of vectors are easy and cheap to produce in large amounts. They are considered safe, as there is no possibility of recombination and have a limited immunogenicity, which allows re-dosing [77, 78].

i. Electroporation

Electroporation is a physical transfection of genes. During electroporation cells are resuspended in an appropriate buffer and undergo a high-voltage current pulse creating nanometre pores in the cell membrane, which allows DNA or mRNA to enter the cell [77, 79] . It is considered a powerful tool for nuclease delivery although the mechanism is not completely understood.

Nucleofection is a type of electroporation that allows the direct delivery of molecules into the nuclei of mammalian cells. The advantage of nucleofection is that it can deliver nucleic acids and proteins into the

nucleus without breaking the nucleus or needing the cell to divide, so it is quite useful for quiescent cells [80]. The successful delivery of ZFNs targeting the human C-C chemokine receptor 5 (CCR5), a co-receptor for HIV entry, into human HSC conferred resistance to the T cells derived from the HSC CCR5 KO against HIV infection [81]. Additionally, the delivery of Cas9 and a donor template into induced pluripotent stem cells (iPSC) produce a mutation in CCR5 that resulted upon iPSC differentiation into monocytes and macrophages resistant to HIV [82]. Improvements in nucleofection have been achieved by avoiding the use of plasmids that cause toxicity in some cell lines and rendered poor efficiency [83]. In this direction, the delivery of ZNF [84] or Cas9 [85] as mRNAs have increased efficiency in HSC, embryonic stem cells (ESC), iPSC, zygotes and T cells [86]. To further improve the delivery of the site-specific nucleases, the removal of the transcription or the translation was achieved by ribonucleoproteins (RNP); where proteins are conjugated with nucleic acids [85]. The introduction of the Cas9 protein/gRNA RNP complex into cells by nucleofection has been tested in HSC [87], T cells [88], iPSC and ESC [89] observing a lower off-target effect. Additionally, this delivery method does not induce the response of the host immune system. However, the amount of Cas9 protein that needs to be delivered in the cells to be effective is very high limiting this approach for *in vivo* applications [90].

ii. Liposomes/polycations

Liposomes, which belong to the lipid-based group [91], are a combination of plasmid DNA and a lipid solution. They fuse with the cell membrane of different cell types and introduce transiently the plasmid DNA into the cytoplasm and nucleus [92]. They are widely used in gene delivery for animals and patient cells, and they have been used in phase I and II clinical trials, such as lipofectamine and lipofectin. Liposomes are made of one or more concentric lipid bilayer membranes giving a hydrophilic space between them. Their morphology and size changes based on the lipid composition, formation conditions, proportion of lipids to genetic material, molecular weight, structure and size of the genetic material [91, 93]. Liposomes have no replication risk and are less immunogenic than viruses.

Cationic liposomes are becoming increasingly prevalent in the field of gene therapy [93]. Polycations are macrolythic molecules. They are amphiphilic, meaning that on one face they have cationic groups that provide the necessary positive charges for the DNA to bind and on the other face they have lipid-type chains which makes them self-aggregate in a defined pattern [94]. Given the negative charge of the cell membrane, cationic liposomes encapsulating genome editing nucleases easily induce their cellular uptake [78]. However, they have not shown positive results in animal models.

b. Viral vectors

Viruses, in nature, have evolved to deliver their genomes into cells efficiently. Thus, recombinant DNA technology has taken this aspect and modified them into a replication-defective virus that carries genes of interest, heterologous (non-viral) genetic information, into a cell, this is called transduction.

The efficiency of the best viral vectors system can reach 100% of the target cells, but of course, this will always depend on the target cell type and on the viral vector [95, 96]. There is no proven one-fits-all viral vector suitable for all cell types. Actually, each viral vector has its own limitations and advantages when being used [97] and the most suitable one should be chosen depending on the target cells. Below we will describe extesnsively retroviral vectors since we have focused our strategies to deliver endonucleases with these vectors systems:

i. Adeno-associated virus (AAV)

The first publication showing efficacy of AAV vectors delivering ZFNs was in 2012. Shortly after that, it was reported that AAV were used to deliver not only ZFNs but also the donor template in a murine model. In CRISPR/Cas9 case, there have been several attempts to incorporate the Cas9 and the gRNA into AAV vectors, giving inconclusive results. However, delivery of gRNA and Cas9 by separate AAVs has been effective in a mouse model of Duchenne muscular dystrophy and liver disease [98]. However, because of its small packaging capacity other viral vectors have been studied for the delivery of the CRISPR/Cas9 system. In any case, the best solution in the context of AAV delivery systems, is to use a smaller Cas9 (*Straphylococus aureas* which is 3.2kb), which is also efficient [99].

In addition to their potential to deliver gene editing tools, AAV vectors have been reported to enhance up to 1000-fold the homologous recombination without nucleases. Some scientist have taken advantage of this property and have combined different methods of delivery of the nucleases with AAV vectors delivering the donor template [98].

ii. Adeno viruses (AdVs)

AdVs belong to the *Adenoviridae* family. They have been modified to allow more packaging capacity, but they still are limited for *in vivo* use by the inflammatory response and a cytotoxic T-cell response [78]. Nevertheless, AdVs have been used to deliver ZFNs to target $CD4^+$ helper T cells from HIV infected patients and were used in clinical trials [100, 101]. AdVs were also applied to deliver TALENs [102] and CRISPR/Cas9. They also have been used as delivery tools of large donor templates, [98].

iii. Retroviral vectors

Retroviruses have a RNA genome, and the appropriate machinery to reverse transcribe it into double stranded DNA, which then will stably integrate into the host genome, is to use the cell's machinery for their own replication and long-term expression [103]. It was 30 years ago when the first evidences demonstrated that retrovirus-based gene transfer was better than DNA transfection and that it was used in bone marrow transplantation in murine models. During these past 30 years a better understanding of the biology of the *Retroviridae* family has been achieved, giving more knowledge for better design of retroviral vectors for specific applications, making them more efficient and safer [104]. The vectors have been designed based on different retroviral family members such as foamy virus [105], human immunodeficiency virus (HIV) [106], simian immunodeficiency virus [107], bovine immunodeficiency virus [108], feline immunodeficiency virus [109], equine infectious anaemia virus [110], murine leukaemia virus (MLV), bovine leukaemia virus [111], rous sarcoma virus [112], spleen necrosis virus [113] and mouse mammary tumour virus [114].

The most used vector in clinical trials is the one derived from the MLV (GV), because it is able to transduce and integrate in a wide range of human cell types and they were the first integrative vectors to be developed. However, it is not able to transduce non-dividing cells such as T cells or hematopoietic stem cells (HSC), among others [115]. LVs on the other hand have overcome this issue and are at this moment the vector of choice for multiple applications.

1. Lentiviral *vectors (LV)*

Lentiviruses receive their name for the long period of time that elapses between the initial infection and the onset of the disease. They have two phases, 1) where the viral genome is inserted into the host cell and 2) when the viral propagation begins [96]. These viruses are able to infect non-dividing cells and transport their DNA into the host nucleus by a mitosis-independent transport mechanism, where it integrates stably allowing a long lasting expression of the transgene. The advantage of these vectors is that they are able to accommodate up to 10kb long transgene expression cassette, although the bigger the insert the lower the titre [116].

The vectors are generated by trans-complementation, where the producing cells are transfected with different plasmids containing the gene of interest and the packaging construct which encodes the essential protein for the lentivirus assembly [117] (Figure 7).

Retroviral vectors (as retroviruses) do not integrate randomly, they prefer active transcription areas. Nevertheless, the integration pattern of the GV which integrates near transcription start sites and DNAase I hypersensitive sites, differed from that of the lentivirus. Although lentiviruses integrate in active transcription units, their integration is more spread within actively transcribed genes and thus further away from the transcription start site [117].

Since the initial vector system was designed, there have been different modifications, but three of them stand out: 1) the 5' U3 viral promoter has been substituted for a Tat-independent transcription promoter; 2) the 3' U3 enhancer/promoter sequence has been deleted, so there is no functional U3 viral promoter given them the name of self-inactivating (SIN) vectors. This modification increases safeness increase safety because of the lack of expression of ψ-bearing mRNA upon integration, eliminating the possibility of

vector mobilisation upon integration and 3) the introduction of Cppt-CTS sequence (*pol* gene-derived sequence) which improves transduction efficiency [96].

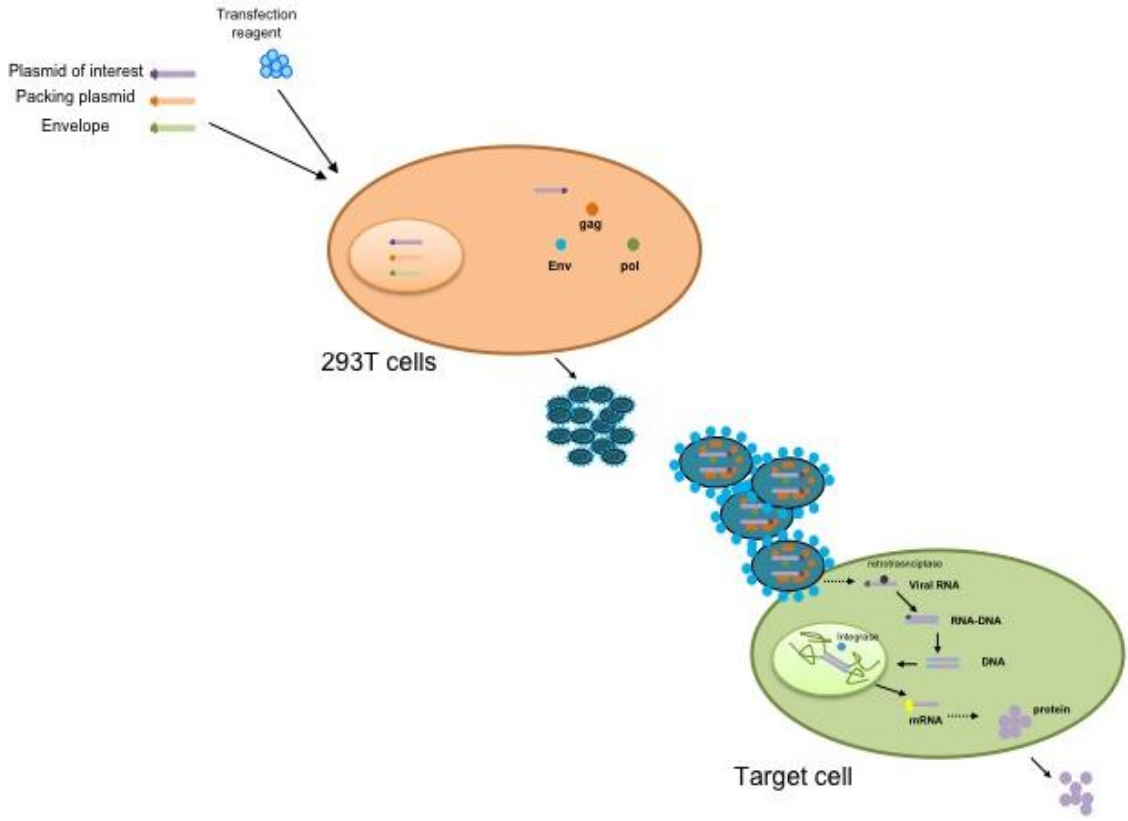


Figure 7. Procedure of Lentiviral production process and transduction of the target cell. Packaging cells (293T) were transfected with the plasmids that codify for envelope (env), gag, pol and the gene of interest (in purple). Once the virions are assembled, the supernatant containing the viral particles is collected and used to infect different cells (target cell).

Other steps to create safer vectors are the introduction of strong RNA polyadenylation sites, so that read-through transcripts are avoided, or the introduction of DNA insulators into the LTR, which improves the internal promoter/enhancer isolation from the genome host and vice versa [116, 117].

Other modifications of LVs include the integration-defective lentiviral vectors (IDLVs) to achieve transient expression of the transgenes in dividing cells or long term expression in quiescent cells. Several IDLVs have been produced by using mutant integrases. After transduction, the IDLVs RNA is reverse-transcribed to a double-stranded DNA (dsDNA) and imported into the nucleus of the transduced cells in the form of 1- or 2-LTR circles. Current research in the field has demonstrated that IDLVs are suitable for different applications both *in vitro* and *in vivo* models ([118], including genome editing).

a) Pseudotyping

Pseudotyping is the production of viral vectors in combination with glycoproteins from other enveloped virus. These glycoproteins provide the vector with its specific tropism [119].

Pseudotyping has been reported to improve vector titers, efficiency of packaging, stability, cellular tropism, immune response and inactivation by complement. To construct a phenotypically mixed particle heterologous, glycoproteins are incorporated into the viral vector which will then emerge from the producer cells. The most notable glycoprotein used in LVs is the vesicular stomatitis virus G (VSV-G) protein, however alternative envelope glycoproteins have been reported to produce safer and more efficient LVs for clinical applications [116, 120, 121].

i. VSV-G

The vesicular stomatitis virus (VSV) is a negative sense RNA virus from the *Rhabdoviridae* family. Its natural hosts are horses, cattle, pigs and other mammals and insects. There are 5 genes which are encoded in the 11kb genome; the G attachment protein enables infection of a wide range of cells [122]. It was only quite recently that the low density lipoprotein receptor (LDL-R) was described as the cell surface receptor that interacts with the G attachment glycoprotein [120, 123]. The broad tropism of the VSVG is due to the wide expression of the LDL-R. In addition, the viral properties in terms of replication and small size are what make the VSV a common virus for basic research being a perfect candidate to confer wide tropism to retroviral vectors.

The VSV-G retroviral pseudotype has proven to be stable and can be obtained at high titers by ultracentrifugation-concentration than other viral envelopes [124]. Currently, most of the LVs are pseudotyped with this envelope to transduce various cell types [125], however, their sensitivity to the human complement system, their toxicity at high concentrations and their pantropic cell recognition have risen safety concerns for their *in vivo* usage [125, 126].

These limitations can be overcome by using glycoproteins from other enveloped viruses.

ii. Feline endogenous retrovirus (RD114)

In 1971 the RD114 virus, a cat endogenous retrovirus, was isolated from the human *rhabdomyosarcoma* cell line [127]. It is a recombinant of the *gag-pol* gene from the cat virus FcEV (*Felis catus* endogenous retrovirus) and the *env* gene from the primate baboon endogenous retrovirus [128].

The RD114 virus envelope glycoprotein recognizes the ASCT2 neutral amino acid transporter [129, 130]. It has been shown that LV RD114 pseudotype transduces efficiently HSCs [131, 132]. Moreover, as the RD114 envelope is not cytotoxic, the particles are stable and it is resistant to the human complement system, being an alternative to VSV-G LV and a promising envelope for *in vivo* application for HSC transduction [131, 133].

iii. Baboon endogenous retrovirus

In 1973 the baboon endogenous retrovirus (BaEv) was isolated from the baboon placenta [134]. Like RD114 virus, the baboon endogenous retrovirus is a chimeric retrovirus, the *gag-pol* genes are from the *Papio cynocephalus* endogenous virus and the *env* gene originates from the simian endogenous retrovirus [128, 135].

The BaEV recognizes not only the ASCT2 receptor, but also additionally the human ASCT1 carrier, which presents 57% of homology with ASCT2 [130].

iv. Measles virus

Measles virus (MV) is an enveloped single stranded RNA virus, which belongs to the *paramyxoviridae* family. The MV genome encodes 8 proteins, including 2 surface glycoproteins; the envelope proteins: Haemaglutinin (H) and the fusion protein F. This virus recognizes several receptors; signalling lymphocyte activation molecule SLAM/CD150 [136, 137] present on T, B and mature dendritic cells; Nectin-4 [138, 139], present in endothelial cells and the complement-regulatory molecule CD46 expressed on all nucleated human cells [140]. The LVs pseudotyped with the H and F glycoproteins have the exceptional capacity to efficiently transduce quiescent human T cells [141], B cells [142, 143] and monocytes derived from dendritic cells [126], offering promising results for gene therapy and immunotherapy [144].

2. Integrative-deficient-Lentiviral vectors (IDLVs)

It is possible to replace the surface glycoproteins by those from other viruses (pseudotyping). They ensure long-term expression and efficient transfer without *in vivo* inflammatory responses and can accommodate sequences up to 10kb. However, it has been reported that they cause insertional mutagenesis causing several cancers in clinical trials [117]. To avoid these possible side effects, IDLVs have been used instead. This is the case of a study where ZFNs and a DNA template were delivered using IDLVs showing efficient delivery into different human cells including HSC and embryonic stem cells [145]. Nevertheless the efficiency of IDLVs varies depending on the target cell. For example IDLV transduction efficiency in mice hepatocytes is lower than that of LVs, while IDLVs are very efficient in mouse muscle [78, 117]. LVs seem to be insufficient to deliver and express TALENS in mammalian cells, apparently due to recombination in

the LV genome caused by the inserted repetitive sequences of the TALENs [146]. Although there has been some successful results with CRISPR/Cas9 encoding LVs in post-mitotic brain neurons[147] and T cells [148]. IDLVs have been reported to gene edit efficiently in immortalized cells [147]. This might be because LVs are normally better than IDLVs in terms of expression levels [149, 150] because IDLVs are prone to epigenetic silencing by chromatinization [151].

In order to improve the expression and titer of IDLVs, there have been different strategies. One of the most successful was the inclusion of insulators [152]. An insulator is a specific DNA sequence and all their associated binding proteins, which help to establish or maintain inter-domain boundaries [153]. They help to form functional boundaries between different chromatin domains [154]. The barrier insulators protect against heterochromatin-mediated silencing (Figure 8). Normally, they are located between promoters and enhancer or heterochromatin, so that they can provide their protective function. The main reason to include the insulators is to avoid silencing of the transgene.

The most frequently used insulators are the ones based on the chicken β-globin locus control regions hypersensitive 4 (HS4). It is through the cis active elements that HS4 increases the episomal efficiency [155], by the interaction of the nuclear proteins CCTTC-binding factor (CTCF) with the nuclear matrix [156], avoiding heterochromatin spreading (barrier insulator) in the episomal DNA. The HS4 insulator activity has been restricted to the 5' 250bp, named "core" [157], and it has been reported that it might be sufficient to add an insulator, although it might depend on the cellular type and the vector. An extended version, 400bp fragment, has been tested too, named "Ext" (Extended) (Figure 8B).

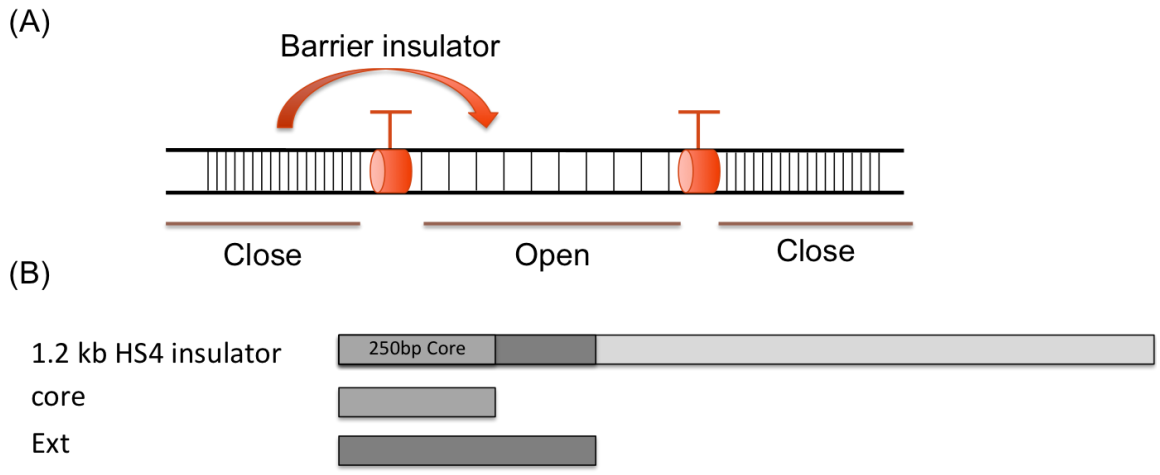


Figure 8. Chromatin insulator function. **A)** Scheme of insulator function; barrier insulators protect against heterochromatin-mediated silencing. Modified from [154] **B)** Scheme from the different HS4 insulators. Top the complete lenght of the HS4, middle the 250bp fragment (core), bottom the extended version (Ext) of 400bp.

Other sequences frequently used as insulators are the scaffold attachment regions (SARs) or matrix attachment regions (MARs). These sequences harbour DNA domains recognized by the nuclear matrix and they organize the chromatin in the nucleus [158, 159]. In this context, it has been shown that the inclusion of the human β-interferon SAR (INF-SAR) element in a retroviral vector inhibited de novo methylation of the retroviral 5' LTR favouring its expression in target cells [158-160].

iv. Virus like particles (VLPs)

Viruses like particles (VLPs) are multiprotein structures similar to natural virions, however; they do not carry a viral genome. Therefore, they are replication and infection incompetent. Normally, they are formed by the assembly of viral proteins, so they are classified depending on the synthesis method or their architecture (enveloped or not enveloped; Figure 9). Because of the multigenicity of the viruses, VLPs

have a wide variety of applications such as vaccines and nanovectors for the delivery of antigens or therapeutic molecules [161].

It was reported in 1979 that VSV-G could be inserted into liposomes *in vitro* [162]. Later, it was observed that VSV-G vesicles are produced and released to the medium without other viral components and can be associated with naked DNA, and these complexes show high transfection efficiency [163].
In addition, it has been reported that the over-expression of VSV-G, alone, promotes the release of particles that contain cellular proteins. The particles can bind and fuse thanks to the properties of the envelope and transfer their protein cargo to other cells such as primary fibroblast and PBMCs [164]. Moreover, the union of gag-pol VLPs with naked DNA and the VSV-G vesicles display higher transfection efficiency [163]. It is in this context that the Cas9 protein can be incorporated to the VLPs for its delivery. For VLPs derived from the bacteriophage P22 the Cas9 was fused to the scaffold protein of P22 and the gRNA was co-expressed with the Cas9 protein [165].

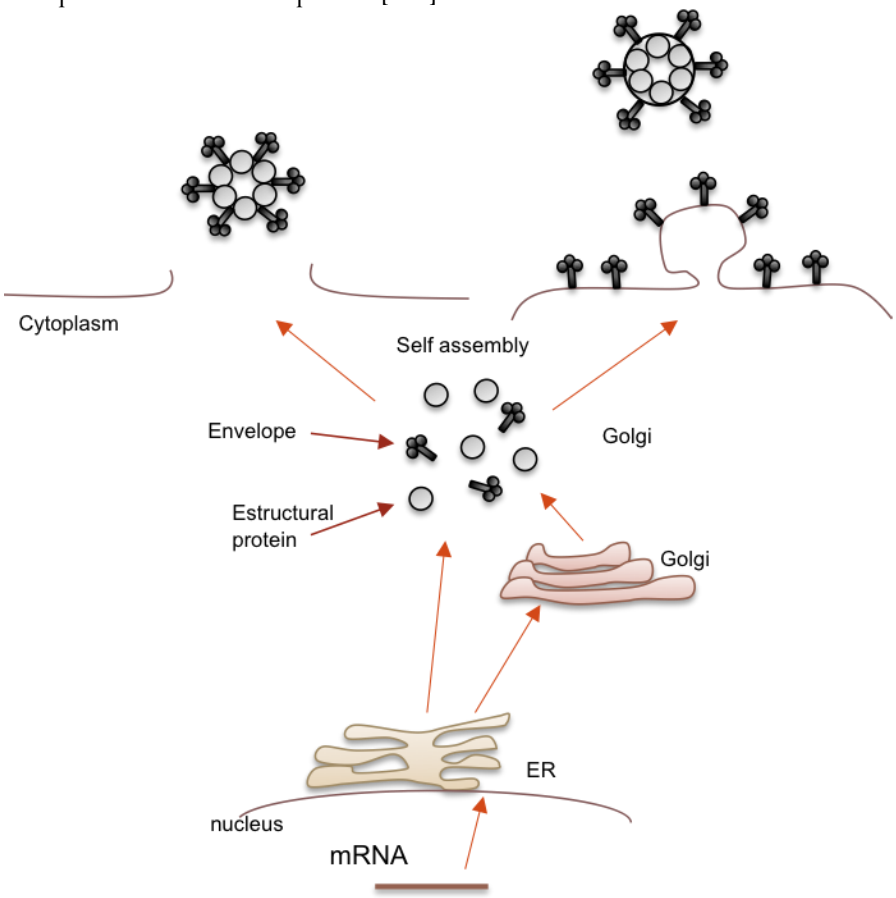


Figure 9. Scheme of non-enveloped (left) and enveloped (right) VLPs formation. Modified from [161]. ER-endoplasmic reticulum:

5. Target cells

a. Haematopoietic stem cells

It was Pappenheim, in 1905, who first described the "stem cell" fate, and his description is still the current approach of haematopoiesis [166]. In 1932, Sabin evoked the presence of undifferentiated hematopoietic stem cells in bone marrow [167]. Some years later, he explored the nature of these stem cells, describing them like small lymphocytes without signs of differentiation. Due to limitations in experimental methods, the debate about the existence of a common hematopoietic stem cell continued for several decades.

Only in the 1960s it was shown that selected subpopulations of haematopoietic cells were able to both self-renew and differentiate into mature haematopoietic lineages [166, 168, 169]. It was also found that splenic

nodules which appeared to be colony forming units (CFU) were composed by all haematopoietic populations [170]. Later, the clonal expansion of the HSC into colony forming units was described [169], providing the scientific basis of haematopoiesis and hematopoietic transplantation. Up to now, human HSCs haven't been univocally characterized by a combination of phenotypic specific markers as it is the case for murine HSCs [171]. Actually, over the last four decades, several methods of human HSC enrichment have been suggested but none have been completely satisfying [172].

The most commonly known human HSC marker, CD34, was identified in 1984 by Civin et al. [173] and it is suggested to play a role in cell adhesion and differentiation. Even though CD34 is the isolation marker by excellence, other markers can be used in order to enrich more primitive HSC subpopulations. This is the case of CD133 (AC133), it is a surface marker expressed on 20 to 60% of the $CD34^+$ isolated cells depending on their origin [174]. $CD133^+$ $CD34^+$ cell subpopulation presents high expansion capacity, bone marrow homing and engraftment [175, 176]. However, CD133 is not a specific marker, in humans it is also found in hepatic [177] and neural stem cells [178]. Another surface marker is CD90 (Thy-1) [179]. Thy-1 is involved in numerous non-immunological processes such as cellular adhesion, tumour growth, migration and cell death [180]. The $CD34^+$ $CD90^+$ subset presents long term repopulation capacity.

Regarding negative markers, the $CD34^+$ $CD38^-$ population consists of a highly primitive subset of HSCs. The increase of $CD38^+$ cells is correlated with the HSC cell cycle entry and their differentiation [181]. Likewise, $CD45RA^-$ $CD34^+$ cells have been reported to be enriched in HSCs [182]. HSCs are also negative for lineage differentiation markers (lin^-).

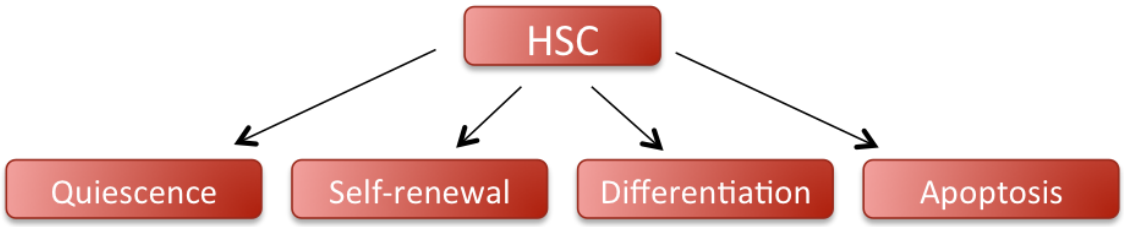


Figure 10. ***HSC cell fate***. HSCs residing in the bone marrow generate mature blood cells throughout the life of organism. A precise regulation occurs to maintain a balance between quiescence, self-renewal and differentiation.

So, the primitive $Lin^-CD34^+CD38^-CD90^+CD45RA^-CD133^+$ cells should be a very enriched subset in HSCs [172]. However, the most used clinical markers at the moment are still CD133 and CD34.

Depending on the hematopoietic conditions, HSCs have several possible fates. They can stay quiescent in their niche, self-renew, differentiate or die through apoptosis (Figure 10). Up to 70% of HSCs are in G0 phase, quiescent, and they are responsible for the functional long term repopulation and engraftment [183]. Long term repopulation potential of quiescent HSCs has been demonstrated by serial transplantation experiments in mice [184]. Multipotency and self-renewal are characteristics of HSCs. Self-renewal allows HSC to proliferate without differentiating and the multipotent character allows them to differentiate into the different lineages. Apoptosis has been described as an important possible fate for HSCs [185]. Actually, defects in HSC differentiation or self-renewal can induce hematopoietic insufficiencies and the development of hematopoietic malignancies [186]. There are cell checkpoint factors, reactive oxygen species, apoptosis specific transcription factors and signalling pathways that act together to maintain the HSC pool [187].

A member of the receptor tyrosine kinase family, Flt3, is commonly used in HSC stimulation assays, as it plays an important role in HSC homeostasis. It is also necessary for preventing spontaneous apoptosis and HSC survival. Stem cell factor (SCF) and thrombopoietin (TPO) are also commonly used in HSC stimulation as they induce survival but their role in apoptosis remains unclear.

b. Human T cells

It is during thymopoiesis that the differentiation of precursors, which have migrated from the bone marrow to the thymus, occurs and gives the T cell repertoire [188] (Figure 11).

The $Lin^-CD34^+CD38^-$ bone marrow subpopulation includes the multipotent early lymphoid progenitors (MLP). They are considered to preferentially differentiate into lymphoid lineages and presumed to contain thymic progenitors [189-191]. The common lymphoid precursor (CLP) is characterized by the expression of $Lin^-CD34^+CD10^+CD24^-$. It has been reported that the $CD24^+$ population is restricted to the B lineage whereas the $CD10^+CD24^-$ can give rise to B, T and NK cells [192]. There is a $CD34^+/CD45RA^{hi}/CD7^+$ population that is able to enter the thymus parenchyma, in *ex vivo* colonization assays, designated as an early T-lineage progenitors (ETP), which constitute the CD4/CD8 double negative subpopulation [193]. The up-regulation of CD7, CXCR4 and CD5 and the down-regulation of CD10 give the pro-T stage characterized by expression of CD34, CD45RA and CD7. The progressive expression of CD5 allows making the distinction between the pro-T1 $CD5^-$ and the pro-T2 $CD5^+$ subset.

At the pre-T stage CD1a appears, which induces the cell into T cell commitment and indicates their capacity to differentiate into NK cells or dendritic cells is lost. In this stage of T cells differentiation rearrangements of the T cell receptor (TCR) β, γ and δ genes are initiated [194] and $CD1a^+/CD34^+$ cells evolve into first $CD4^+$ immature single positive cells (ISP), which contains precursors for the αβ and γδ T cells lineages [195], which then mature into $CD4^+/CD8^+$ $\alpha^+\beta^+$ cells, first by up-regulating CD4 and then CD8 α^+ and CD8 β^+. That is when TCR β-selection occurs in the $CD4^+/CD8^+$ $\alpha^+\beta^-$ and $CD4^+/CD8^+$ $\alpha^+\beta^+$ double positive (DP) subpopulation [196, 197].

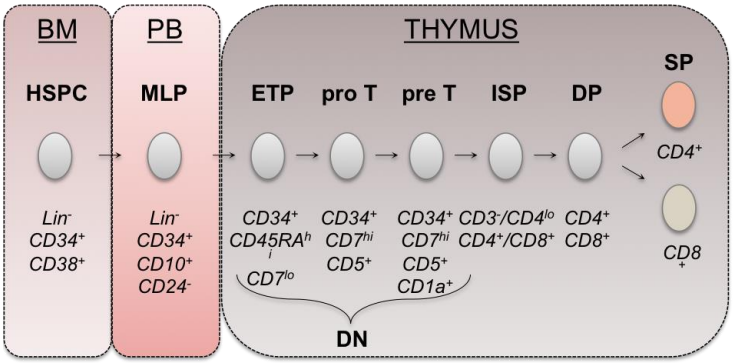


Figure 11. Early stages of T cell development. Schematic representation of human stem cell migration to the thymus and thymocyte development. HSCs from the bone marrow give rise to early thymic progenitors (ETPs), which still have some potential to differentiate into B cell and dendritic cells. The ETPs become double negative (DN) and begin to undergo crucial rearrangement of TCR genes. The TCR gene selection leads to the generation of double positive (DP) cells which express a properly rearranged TCR and both CD4 and CD8 co-receptor. Cells with TCRs that bind to MHC class I molecules retain expression of CD8 and lose that of CD4, whereas cells that bind to MHC class II molecules retain CD4 and lose CD8. For each differentiation stage phenotypic surface markers are indicated. Human stem progenitor cells (HSPC); multipotent early lymphocyte progenitor (MLP); early thymocyte progenitor (ETP); immature single positive T cell (ISP); double positive (DP); mature single positive T cell (SP). Modified from [195, 198].

Once the TCR rearrangements are accomplished, αβ T cells interact with major histocompatibility complex (MHC) antigen expressed on the thymic epithelial cells to survive, and they have to do it with low affinity. Double positive (DP) $CD3^+CD4^+CD8^+$ thymocytes will differentiate into single positive (SP) $CD4^+$ or $CD8^+$ cells due to the CD69 up-regulation and RAG1 down-regulation along with other cell markers [195, 199, 200]. Once they have functionally maturated, CD69 expression decreases and they become $CD4RO^-/CD45RA^+$ [200].

Once the cells have maturated in the thymus into $CD4^+$ and $CD8^+$ SP cells they will migrate to the peripheral blood (PB), where they further differentiate into different subpopulations.

It has been reported that there is a subset of memory T cells. Their generation begins in secondary lymphoid organs when the TCR of naïve cells binds to the major histocompatibility class II (MHCII) on antigen presenting cells (APCs) [201], and then by CD28 and other co-stimulatory signals they switch into effector cells [202, 203].

T cells can be activated by two different pathways *in vitro*:

1) **Stimulation of the TCR receptor**: The TCR is a heterodimeric protein anchored to the membrane consisting of the variable αβ chains expressed as part of the complex with the CD3 protein, which transduce the signals [204]. For T cell activation, TCR stimulation alone is insufficient if not helped by a co-stimulation. The most efficient co-ligand to promote proliferation, survival, cytokine production and metabolism is CD28 [205]. The protocol for *in vitro* activation is based on anti-CD3 and anti-CD28 microbeads and IL-2, which would be sufficient to obtain TCR activated T cells rapidly [206, 207].

2) **Stimulation with IL-7**: IL7 is used in order to stimulate T cells without altering the T population (e.g. naïve T cells [206, 208-210]. IL-7 supports T cell survival and homeostatic proliferation [211, 212]. On top of that, it improves immune reconstitution after allogeneic bone marrow transplantation in mice [213]. Moreover, the proliferation and survival by the treatment with IL7 will not induce the switch from naïve to memory subsets [214, 215] and although it induces up-regulation of different receptors (CD25, CD98, CD71, CD11a and CD40-L [216]), this is not as significant as when stimulating the TCR.

c. Gene editing in primary cells

i. Haematopoietic stem cells

HSCs appear as the desirable target cells for gene editing as they are one of the main target cells for gene therapy. Any modification in the HSC genome will be transferred to all the derived lineages [217]. The efficiency varies with the cell type and cell state. However, it has been reported that adult primary cells use the error-prone NHEJ pathway more frequently that the error-free pathway (HR), because HSC do not divide and this means that HR will probably not work. This imbalance is what makes it a challenge to treat diseases when a gene correction is needed [74]. However, the HR frequency can be increased by inhibiting the NHEJ pathway, although the secondary effects of this inhibition have yet to be studied more thoroughly [217, 218].

Different studies in gene therapy for blood disorders have shown that once the cells have been modified, *in vivo* they can be outcompeted by the residual unmodified HSCs or they diminished their capacity of engraftment. That is why the best diseases to target would be those, for which the corrected HSCs or the HSC derived cells (T and B cells for example) possess a selective advantage over the unmodified one, such as X-SCID, Fanconi anaemia and WAS.

One of the most studied diseases to apply gene editing has been HIV infection [74]. It has been well documented that targeting chemokine (C-C motif) receptor 5 (CCR5), which is the major co-receptor of HIV, is a well-known strategy for the protection against HIV infection. The ablation of CCR5 in HSC results in derived $CD4^+$ T cells resistant to HIV. So this provides HIV infected patients long lasting immunity to the infection, since this strategy protects all lymphoid and myeloid cell types, as they are both targets for HIV infection [2].

Due to the fact that the genome editing through "hit and run" process, they would only need a transient expression, this is one of the main reasons why non-viral methods are attractive, such as RNA nucleofection. However, nucleofection has been associated with high toxicity and loss of engraftment potential in HSCs. Nevertheless, with improved transfection conditions Holt *et al*. were able to have a high efficient disruption of CCR5 by ZFNs nucleofection while limiting the toxicity, and these ZFNs-treated HSCs engrafted in NSG mice as efficiently as untreated HSCs [81]. Indeed, validity of this CCR5 Knock

Out strategy was shown in mouse models and is currently evaluated in a clinical trial using Zinc fingers nucleases against CCR5 [219].

The same delivery system, RNA nucleofection, has been used with CRISPR/Cas9 to target the same gene. When using only one gRNA, the cleavage percentage is similar to that obtained by nucleofection with ZFNs, but when using multiple gRNA, the percentage of cleaving is 10-fold increased. In this case, no mouse engraftment studies were performed, but CRISPR/Cas9 mediated disruption of CCR5 in $CD34^+$ cells showed that there were no significantly differences in the formation of $CD34^+$ cell derived CFU myeloid colonies [2, 220].

Another gene that has been targeted in HSCs is the interleukin-2 receptor gamma (IL2RG); its mutation causes an X-liked severe combined immunodeficiency (SCID-X) [84, 221]. To target this gene, ZFNs have been introduced by nucleofection of the mRNA and the donor DNA was introduced as an IDLV. Genovese *et al*. have optimized a protocol to gene edit IL2RG in HSCs efficiently. They have observed a functional reconstitution of IL2RG in the lymphoid progeny of HSCs that was able to repopulate transplanted mice, which confirmed the ability to edit long-term repopulating HSCs [84].

ii. T cells

Using peripheral blood T cells is advantageous for several reasons; 1) they are more accessible for genetic modifications than other target cells, such as HSCs, 2) they can also be isolated in high amounts, 3) they have lower risk of transformation and 4) long term correction can be achieved by T cell therapy, as naïve cells which respond to a novel antigen can persist over years in patients, in several genetic dysfunctions of the hematopoietic system such as immunodeficiencies (eg SCID), cancers and acquired immunodeficiencies infections [222-224].

Actually gene editing for therapeutic purposes is being applied in cancer immunotherapy, mainly by the production of next-generation chimeric antigen receptors (CAR) T cells. Basically, CARs mix extracellular single-change variable fragments specific to an antigen on tumour cells with intracellular chimeric signalling domain that drives T cell activation [225]. The ideal would be to use autologous T cells, but the low quantity and quality of the cells as well as the expenses of the manufacturing might prevent of its full potential. This is why CAR T cell therapy could benefit of an "universal" CAR T cells. Because endogenous TCR could lead to graft-versus-host disease (GvHD), different groups have used genome editing to knockout the TCRα/β chains by ZFNs, TALENs and CRIPSR/Cas9 nucleases efficiently [226]. When combining this "universal" T cell with different disruptions of other genes (B2M and PD-1) by Cas9 m-RNA, they had a high anti-leukemic activity, reduced alloreactivity and did not induce GvHD in NSG mice [227].

Another targeted disease is HIV infection. After the "Berlin patient", where the transplantation of homozygous CCR5 mutated conferred resistance to HIV, there have been different studies to achieve the same result. ZFNs have been used to knockout CRR5 expression on T cells. These *ex vivo* modified T cells were injected into patients and were seen to persist longer than the unmodified ones, suggesting that CCR5 KO confers a survival advantage. TALENs have also reported a high percentage of CCR5 disruption, showing even lower off-target effects at the CCR2 locus as well as lower cytotoxicity. CRISPR/Cas9 has also been used in studies with human primary T cells, although in this case it is the CXCR4 locus that has been targeted in order to achieve T cells resistant to the X4-tropic HIV strain. In summary, T cells present an important therapeutic potential, either for autologous or allogenic populations

HIPÓTESIS Y OBJETIVOS / HYPOTHESIS AND OBJECTIVES

"The grand aim of all science is to cover the greatest number of empirical facts by logical deduction from the smallest number of hypotheses or axioms."

Albert Einstein

Hipótesis

La edición génica podría mejorar la terapia génica actual para el síndrome de Wiskott-Aldrich debido al aumento de la seguridad, ya que está dirigida a corregir el gen endógeno mutado de *WAS* y podría además aumentar la eficiencia, ya que la expresión del transgen es llevada a cabo por el promotor endógeno de WAS expresándolo fisiológicamente.

Objetivos

El objetivo principal de este estudio es mejorar las herramientas para la terapia génica del síndrome de Wiskott-Aldrich utilizando las herramientas de edición génica mediante la comparación de las diferentes nucleasas y los diferentes sistemas de entrega.

Objetivos específicos:

1. Desarrollar un sistema CRISPR/Cas-9 eficiente frente al locus *WAS* en la misma zona que las ZFNs específicas para WAS desarrolladas por Sangamo BioSciences, Inc.

2. Comparar la eficiencia y la especificidad de las ZFNs y el sistema CRISPR/Cas9 para la disrupción génica de locus *WAS* utilizando nucleofección e IDLVs.
    a. Células K562
    b. HSCs ($CD34^+$)

3. Comparar la eficiencia y especificidad de la recombinación homóloga de la inserción génica en K562 utilizando ZFNs y CRISPR/Cas9 específicos de *WAS*.

4. Mejoras del envío del sistema CRISPR/Cas9:
    a. IDLVs
        i. Mejora del *backbone* de IDLVs
        ii. Mejora del pseudotipaje de IDLVs
    b. Envío de proteínas-gRNA: Nanoblades
        i. HSCs ($CD34^+$)
        ii. Células T

Hypothesis

Gene edition could improve actual gene therapy for WAS due to increased safety, as it targets and corrects the mutated gene at the *WAS* endogenous locus and might increase efficacy, since the expression of the corrected transgene will be under the control of the WAS endogenous promoter and thus physiologically expressed.

Objectives

The main aim of this study was to improve the tools for WAS gene therapy using gene editing tools by comparing different nucleases and delivery systems.

Specific Aims:

1. To develop efficient CRISPR/Cas9 systems targeting the *WAS* locus at the same location that *WAS*-specific ZFNs available through Sangamo BioSciences, Inc.

2. To compare efficiency and specificity of ZFNs and CRISPR/Cas9 systems for gene disruption of the *WAS* locus using nucleofection and IDLVs.
    a. K562 cells
    b. HSCs ($CD34^+$)

3. To compare efficiency and specificity of homologous-directed gene insertion in K562 cells using *WAS*-specific ZFNs and CRISPR/Cas9.

4. The improvement of the CRISPR/Cas9 system delivery:
    a. IDLVs
        i. Improving IDLVs backbone
        ii. Improving IDLVs pseudotypes.
    b. Protein delivery-gRNA: Nanoblades
        i. HSCs ($CD34^+$)
        ii. T cells

Material and methods

"Our own genomes carry the story of evolution, written in DNA, the language of molecular genetics, and the narrative is unmistakable."

Kenneth R. Miller

Material and methods

1. Cells lines and primary cells

1.1. 293T cells

The 293T cells (CRL11268; American Type Culture Collection; Rockville, MD) are a human embryonic kidney epithelial cell line, which has been modified by the insertion of a temperature sensitive gene codified by the simian SV40 T antigen. This antigen is constitutively expressed to achieve higher transfection efficiency. It also allows the episomal replication of plasmids that contain the replication origin. 293T cells are used to produce non-replicative lentiviral vectors.

They are maintained in Dulbelcco's Modified Eagle's Medium (DMEM, Invitrogen, Edinburgh, Scotland)) supplemented with 10 % Fetal Bovine Serum (FBS) (PAA Laboratories GmbH, Austria) at 37°C, 21% O_2, 10% CO_2.

1.2. K562 cells

The human erythroleukaemic cell line K562 was obtained from ATCC (American Type Culture Collection, Manassas, VA; CCL-243), they lack the MHC complex and any traces of Epstein-Barr virus or other herpes viruses. They exhibit the Philadelphia chromosome [228] and another translocation between chromosome 15 and 17. They are maintained in RPMI 1640 media (Gibco-BRL, Middlesex, UK)), supplemented with 10% FBS (PAA Laboratories GmbH, Austria) [229] at 37°C, 21% O_2, 5% CO_2.

1.3. $CD34^+$ cells derived from umbilical cord blood

Hematopoietic stem cells were obtained from healthy donors cord blood, derived from C-section or natural birth. The blood was diluted in 1:2 of PBS and the mononuclear fraction was obtained by a Ficoll Hypaque (GE Healthcare, Fairfield, USA) density gradient. Erythrocytes were eliminated by quick lysis solution (Cytognos) and the platelets by centrifugation at low speed. The $CD34^+$ population was purified by double elution using the autoMacs Pro separator (Milteny Biotec) after incubation with hCD34 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the company's instructions. To assess the degree of enrichment by CD34 antigen detection we use an anti CD34-Pe-cyanine7 (eBioscience) in BD FACSCANTO II (BD Bioscience) obtaining over 95% purity.

They were maintained and stimulated in Stem Span media (Stem cell), supplemented with 10% of penicillin and streptomycin, stem cell factor (SCF) 100ng/ml (Promega, Fitchburg, Wisconsin, USA), FMS-like tyrosine kinase 3 ligand (Flt3-L) 100ng/ml (Promega, Fitchburg, Wisconsin, USA), thrombopoietin (TPO) 20ng/ml (promega), interleukin 6 (IL-6) 20ng/ml (promega). For expansion, we added StemReginin (SR1) 1µM (Cayman Chemical Ann Arbor MI, USA), UM171 (Apexbio, Boston MA USA) and 35mM 16-16-dimethyl prostaglandin E2 (dmPGE2) 10 µM (Cayman Chemical Ann Arbor MI, USA) after isolation and before transduction of HSCs at 37°C, 21% O_2, 5% CO_2.

1.4. Human primary lymphocytes

Peripheral blood samples, obtained from healthy donors after informed consent, were collected into bags containing anticoagulant. Peripheral Blood Mononuclear cells (PBMCs) were isolated upon Ficoll gradient (Sigma-Aldrich, St Louis, MO, USA). $CD19^+$ B cells and $CD3^+$ T cells were purified by depletion using the B cell isolation Kit II (Miltenyi Biotec) for $CD19^+$ B cells and the Pan T cells isolation kit (Miltenyi Biotec) for the $CD3^+$ T cells and separated by the Automacs pro-separator (Miltenyi Biotec). Purity of isolated B- and T-cells was monitored using anti-hCD19APC and anti-hCD3PE antibodies, respectively, and was analyzed by flow cytometry (MACSQuant VYB, Milteny Biotech).

Freshly isolated unstimulated T and B lymphocytes were seeded in RPMI 1640 medium (Gibco Invitrogen, Auckland, New Zeland) supplemented with 10% FSC (Lonza, Verviers, Belgium) and penicillin/streptomycin (Gibco, Invitrogen, Auckland, New Zealand). B cells were stimulated for 24h with Pansorbin and IL2 (2µl/2ml and 100ng/ml respectively). T cell were activated for 3 days either by IL7 (20ng/ml) or by stimulation of the TCR using the TransAct CD3/CD28 kit (40µl) (Miltenyi Biotech) and IL2 (100ng/ml) in RPMI 1640 medium supplemented as previously described [206].

2. Plasmids

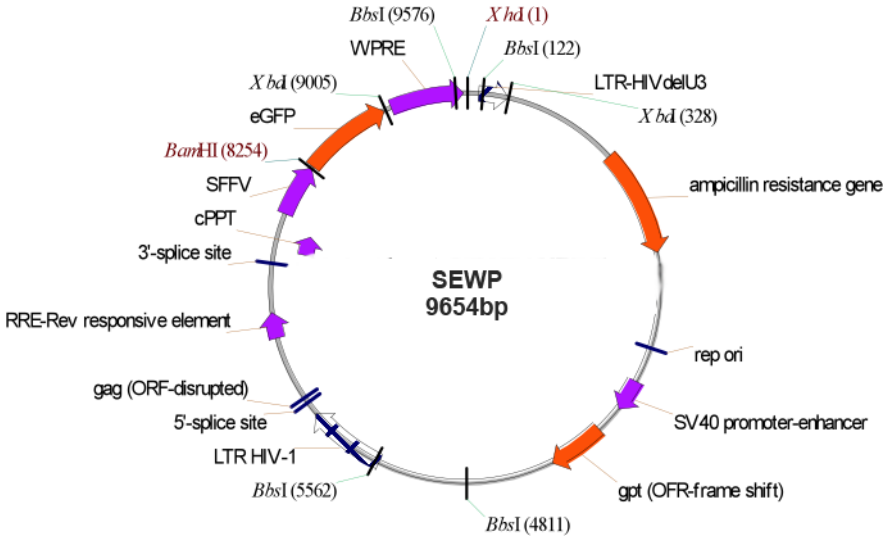


Figure 12. Scheme of the vector SEWP. The spleen focus forming virus (SFFV) promoter drives expression of an eGFP reporter gene. WPRE, the woodchuck hepatitis virus post-transcriptional regulatory element.

The lentiviral vector backbone used in this study was a SIN lentiviral vector. The plasmid from which all cloning where made was the SEWP which contains an enhanced green fluorescence protein (eGFP) gene under the control of an internal spleen focus-forming virus (SFFV) promoter and Woodchuk Hepatitis virus (WHP) Posttrancriptional Regulatory element (WPRE) (Figure 12).

2.1. Zinc Finger nucleases (ZFNs)

Specific ZFNs to target *WAS* locus was incorporated into a SIN lentiviral vector under control of the SFFV promoter. ZFNs targeting the *WAS* locus ZFNs_wt (pVAX-N2A-3FN-15724fok and pVAX-N2A-3FN-15755fok) and ZFNs_mt (pVAX-N2A-3FN-15724fokEL and pVAX-N2A-3FN-15755fokKK) were designed using an archive of prevalidated zinc finger modules by Sangamo Bioscience (Richmond, California, USA), as previously described [63, 64, 230], and kindly provided by Dr. Michael Holmes (Sangamo BioScience). Both vectors targeted a sequence in the first intron of the *WAS* gene and the ZFNs mt contained a mutated version of Fok1 nuclease that increases the affinity by heterodimerization.
To introduce the different monomers of the ZFNs under the SFFV promoter in the SEWP, both SEWP and pVAX vectors were digested with BamH1-Sbf1 and EcoRI-Sbfi respectively and were blunt ended using klenow enzyme, fragment corresponding to ZFNs monomores, and backbone corresponding to SEWP, were ligated. The obtained construct were sequenced

2.2. CRISPR/Cas9 system

The Cas9 nuclease cDNA was obtained through gene synthesis (GenScript: plasmid pUC-coCas9nl2x) of a human codon-optimized cDNA version of Streptococcus pyogenes M1 Cas9 sequence (ref AE004092.2, from nt 854751 to 858857), further modified to eliminate the restriction sites for *EcoRI*, *ClaI*, *BamHI*, *NotI*, *MluI*, *BmgBI*, *AscI*, *AarI*, *AsiI*, *PstI*, *SbfI*, *XhoI*, *SciI*, *KpnI* and *XbaI* and to introduce a *BstX1* site in the

+43 position. In addition, a sequence containing a HA tag and two NLS signals from SV40T were included at the 3'. Finally, the coCas9-HA-nls2 sequence was flanked by a *BamH1/AscI site* at the 5' and *PST/AarI* site at the 3′to facilitate cloning into a lentiviral plasmids backbone.

Guide-RNA (gRNA) were designed by searching for protospacer adjacent motives (PAM) sequences near the cutting site of the *WAS*-specific ZFNs. BLAST searches of the different gRNAs come out with only one gRNAs (gRNA9: 5′-GAGGCAGGAAGGACCAGGTC-3′) with good characteristics (target the same region as the ZFNs and have no homology with other regions in the genome). The gRNA9 was synthesized by GenScript (GenScript Biotech Corporation, Piscataway Township, NJ, USA) together with the scaffold RNA and the U6 promoter to ensure a robust expression of the gRNA molecule (plasmid pU6_gRNA9 containing the BbsI-U6-gRNA9-scaffold-BbsI)(Figure 13A). In order to include a new restriction site (EcoR1) a PCR was performed using U6-gRNA9-scaffold as a template, the PCR products were cloned in pGEMTeasy vector. The resulting constructs were sequenced before use (Figure 13B).

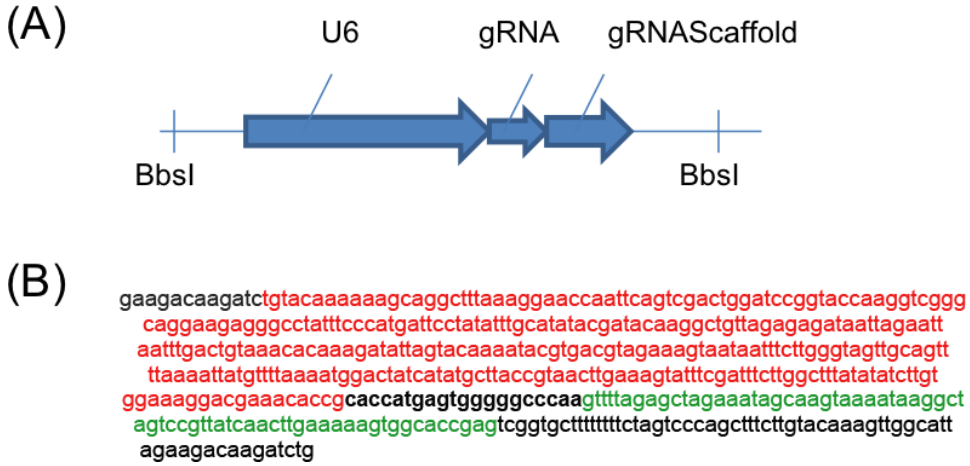


Figure 13. Plasmids harboring the U6 promoter, the gRNA and the gRNA scaffold flanked by BbsI restriction site. **A)** Scheme of the U6-gRNA-gRNAscaffood region. **B)** Sequence of the U6 (red)-gRNA (bold) -gRNA scaffold (green) region.

SELg9 and SELg9WP plasmids were obtained as followed: A *XbaI* fragment of the SEWP plasmid containing the 3'LTR was subcloned into a pUC19 plasmid to obtain the pUC19LTR. The gRNA9scaffold was introduced into the 3' LTR *Bbs1* site of the pUC19 by standard molecular techniques to obtain the pUC19-LTR-gRNA9. Finally, an *XbaI* fragment of the pUC19-LTR-gRNA9 containing the LTR-gRNA9 was used to replace the XbaI fragment of the SE and SEWP lentiviral vectors to obtain the SELg9 and SELg9WP plasmids.

To obtain the SCas9Lg9 and the SCas9Lg9WP, the eGFP cDNA from the SELg9 and SELg9WP were removed by *BamHI-PstI* and replaced by a *BamHI-PstI* fragment from the pUC-coCas9nls2x plasmid containing the codon optimized Cas9 cDNA.
To obtain the SCas9Xg9WP and the SCas9Eg9WP plasmids, the gRNA9 was amplified by PCR with primers containing the *XhoI* or EcoRI sites (see Table A) and inserted into the XhoI and EcoRI (sites of the SCas9WP respectively.

All the constructs generated were verified by automated DNA sequence analysis and with multiple restriction enzyme digestions.

Primer's name	Sequence
XhoI_U6	CCTCGAGGAAGACAAGATC
XhoBsiwI_gRNA_Rev	CTCGAGCGTACGTAATGCCAACTTTG
EcoI_U6_Fw	TATAGAATTCAGGAAGACAAGATC
EcoI_gRNA	ATATGAATTTACGTAATGCCAACTTTG

Table A. List of primer used for cloning.

2.3. Envelopes

The baboon endogenous retrovirus (BaEV) envelope glycoproteins were previously described [231-233] [234]. Briefly, the cytoplasmic tail of the wild type BaEV was replaced by MLV-A, resulting in the BaEVTR-gp, and the deletion of the R peptide sequence gave the BaEVRless-gp (BRL) (Figure 14A). All envelope glycoproteins were expressed in the phCMV-G expression plasmid [235].

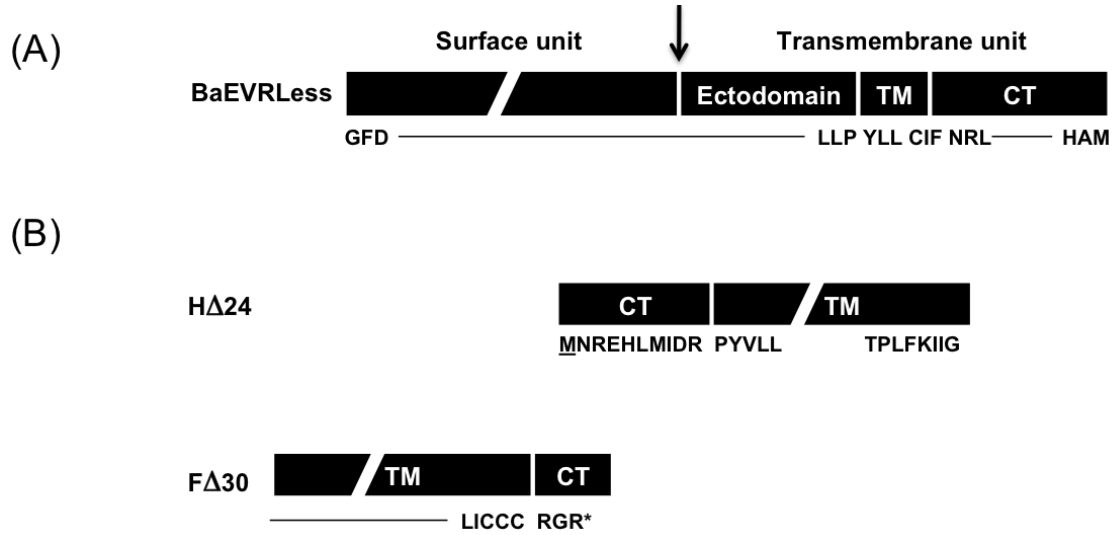


Figure 14. Schematic representation of the envelope glycoprotein. A) Schematic representation of the mutant BaEV (BaEVRless-BRL). The 17 amino acid long cytoplasmic domain was exchanged for the one of the MLV-A glycoprotein resulting in the chimeric BaEVTR-gps, and the R-peptide of the cytoplasmic tail of BaEVwt was deleted resulting in the BaEVRLess mutant gp. **B)** Schematic presentation of mutant F and H gps from the Edmonston MV strain. HD24 is an H mutant with deletion of the cytoplasmic tail. FD30 contains only 3 residual amino acids of the F wt cytoplasmic tail. CT: cytoplasmic tail, TM: transmembrane. (Modified from [234]).

Edmonston's Glycoproteins H and F were inserted into pCG plasmids under the control of the cytomegalovirus early promoter. The plasmids pCG-HΔ24 and pCG-FΔ30 code for cytoplasmic-tail mutants of H and F, respectively (Figure 14B) [142].

3. Vector production

3.1. Production of non-integrating lentiviral vectors

LV were produced by co-transfection of 293T cells with three plasmids; 1) vector plasmid (SEWP, SC9WP, SELg9, SC9Lg9, SC9Lg9WP, Eg9SC9WP,SC9Xg9WP SC9Xg6Lg9, SZFN24WP, SZFN55WP), 2) the human immunodeficiency virus (HIV) packaging plasmid pCMvdR8.74D64V and 3) the envelope plasmid (VSV-G (Pmd2.G)) [15], the HF, BRL or a mixture of both envelope plamids (VSV-G (Pmd2.G) and BRL). For VSVG, vector production was performed as previously described [229]. Briefly, 293 T cells were plated on amine-coated petri-dishes (Sarsted, Newton, NC), in order to ensure the 80-90 % of confluence for transfection. The vector, the packing pCMvdR8.74D64V and the envelope plasmid, were

resuspended in LipoD293 (SignaGen, Gainthersburg, MD, USA), the proportion used is described in table B. The plasmid-LipoD mixture was added to pre-washed cells and incubated for 6-8h, when new medium was added. After 48 hours viral supernatants were collected, filtered through 0.45 µm filter (Nalgen, Rochester, NY) and concentrated either by ultrafiltration at 2000xg at 4°C, using 100KD centrifugal filter devices (Amicon Ultra-15, Millipore, Billerica, MA) or ultracentrifugation (Beckman, 23000rpm 2h at 4°C). Concentrated vectors were either directly used or aliquot and frozen at – 80°C.

Vector production with the different envelopes for primary cells was performed as previously described [231]. Briefly, self-inactivating HIV-1- derived vectors were generated by transiently transfection of 293T cells. For display of BRL-gps 7µg of envelope gp-encoding plasmids were transfected for BRL-LV production. For HF-LV production the co-display of the different H and F gps was needed and 3 µg of each envelope plasmid was used. 5 and 2 µg were used for the co-display of BRL and VSVG envelopes respectively for the BRL+VSV-G- LV production; 3 µg of VSVG-Ggp encoding plasmid was used for VSV-G-LV production.

plasmid	proportion
Vector	5
pCMvdR8.74D64V	2.5
VSV-G	1.5
Lipo D	60µl

Table B. Proportions used for IDLV vectors depending on the envelope vector and Lipo D quantity

The different envelopes were transfected with a gag-pol packaging plasmid and a plasmid encoding either a vector expressing GFP or Cas9 with the gRNA (SC9Lg9W or Eg9SC9W). For integration deficient lentiviral vectors (IDLV) the packaging plasmid was different having a mutation (D64V) in the integrase, making the expression transient. The mixture of all the plasmids was combined with water and the $CaCl_2$ (Sigma-Aldrich, St Louis, MO), then this mix was added to the Hepes buffer saline (HBS) buffer (20mM Hepes, 280mM NaCl, 10mM KCl, 1.5mM Na_2HPO_4, 12Nm D-glc pH 7). After a5 minute incubation, the mixture was added to the cells. Medium was replaced by Optimen supplemented with Hepes (Gibco, Invitrogen, Auckland, New Zeland) and penicillin/streptomycin (Gibco, Invitrogen, Auckland, New Zeland) 18h post-transfection. Viral supernatant was harvest 48h post-transfection, centrifuged and filtered. Low speed concentration of the vector was performed overnight at 3000g at 4°C, and the concentrated virus (100x) were collected the following morning.

Concentrated vectors were either directly used or aliquot and frozen at -80°C.

3.2 Production of nanoblades/VLPs

VLPs (nanoblades) production for primary cells was performed similarly to viral production. Briefly, VLPs particles were generated by transient transfection of 293T cells. For the expression of the Cas9 combined with the gag plasmid, either for the MLV background (BicCas9) or the HIV background (KLP229) 3µg was added.

For display of BRL-gps 5µg of envelope gp-encoding plasmids were transfected for BRL-LV production, for HF-LV production the co-display of the different H and F gps was needed and 3 µg of each envelope plasmid was used. For the BRL+VSV-G- VLPs production 2 µg of each envelope was used; 5 µg of VSVG-Ggp encoding plasmid was used for VSV-G-VLP production. 3 µg of each gRNA (301-

agcctcgccagagaagacaa and 305- gatgcttggacgaaaatgct) was added as well as 3 µg of the gag-pol plasmid either for HIV (PAX2) or MLV (5349). The mixture of all plasmids was combined with water and then, $CaCl_2$ (Sigma-Aldrich, St Louis, MO) and was added to Hepes buffer saline (HBS) buffer (20mM Hepes, 280mM NaCl, 10mM KCl, 1.5mM Na_2HPO_4, 12Nm D-glc pH 7).

A

plasmid	µg
Cas9	3
gRNA	3
BicCas9 or KLP229	3
VSV-G or BRL	5

B

plasmid	µg
Cas9	3
gRNA	3
BicCas9 or KLP229	3
VSV-G	2
BRL	2

C

Plasmid	µg
Cas9	3
gRNA	3
BicCas9	3
H and F	3

Table C. Quantities used for VLP particle production. **A)** Quantities for display of BRL- or VSV-G-gps envelope. **B)** Quantities for display of BRL and VSV-G-gps. **C)** Quantities for display of H and F-gps.

After 5 minute incubation, the mixture was added to the cells. Medium was replaced by Optimen supplemented with Hepes (Gibco, Invitrogen, Auckland, New Zeland) and penicillin/streptomycin (Gibco, Invitrogen, Auckland, New Zeland) 18h post-transfection. Viral supernatant was harvest 48h post-transfection, centrifuged and filtered. Low speed concentration of the vector was performed overnight at 3000g at 4°C, and the concentrated virus (100x) were collected the following morning.

4. Viral titration

Viral titers were determined by the transduction of $1x10^5$ K562 cells/well seeded in 48-well tissue culture plates (BD Biosciences) with four-fold serial dilution of the supernatants and then assaying the percentage of eGFP by FACS analysis 5 days for LVs or 48h for IDLVs after transduction. The LVs and IDLVs particle numbers per ml were also determined using lentivirus titration kit (qPCR lentivirus Titration kit, Applied Biological Materials (ABM)) according to manufactures protocol. Briefly, samples were diluted 1:100 and 1:1000 in 100 µl in PBS prior to lysis. Viral lysis was performed using 2 µl of diluted viral supernatant added to 18 µl Virus Lysis Buffer provided. The qRT-PCR program was performed on an Applied Biosystems 7300-Real Time PCR system using the following qRT-PCR parameters: 1 cycle of reverse transcription (42 °C for 20 minutes), 1 cycle of enzymatic activation (95 °C for 10 minutes), 40 cycles of denaturation (95 °C for 15 seconds) and 40 cycles of annealing/extension (60 °C for 1 minute).

5. Cell transduction

293T and k562 cells were washed with with Dulbecco's PBS (1x) (PAA Laboratories GmbH, Austria) then only the 293T were dissociated with TrypLE Express (Invitrogen), centrifuged at 1250rpm for 5 minutes and counted. 293T and K562 $2x10^5$ cells were plated in 6-well plates (Costar, Corning incorporated, NY) with DMEM or 24 well plates with RPMI respectively.

To transduce primary cells with the different pseudotypes, the day before transduction, 48-well plates (BD Bioscience) were coated with retronectin (Fisher Scientific). The day of transduction for GFP vectors, $5x10^4$ $CD34^+$ cells, $2x10^5$ quiescent and $1x10^5$ activated B- and T- cells were seeded and concentrated vectors added in a MOI (multiplicity of infection) 10 for HF and BRL vectors and MOI 50 for VSVG and BRL+VSVG vectors. The percentage of GFP^+ cells was analyzed 24, 48 and 72h after transduction for B, $CD34^+$ and T cells and additionally at day 6 for $CD34^+$ and T cells, by flow cytometry (MACSQuant VYB, Milteny Biotech). For Cas9 vectors, $5x10^5$ cells were used in 48-well-plates coated with retronectin and concentrated vectors were added, after 24h cells were washed, fresh media and vectors were added again. Cells were pelleted 48h post-transduction for their subsequent DNA extraction and quantification of cleavage efficiency.

For transduction with VLPs, 48-well plates were coated with retronectin the day before, $5x10^5$ cells were used in a final volume of 200µl and the VLPs were added. After 5h of incubation new fresh medium was added to the cells and 24h post-transduction the cells were pelleted followed by DNA extraction and quantification of the cleavage efficiency.

6. Preparation of cells for flow cytometry

Controls and transduced K562 cells (1-4 x 10^5) were collected and washed with cold FACS buffer (PBS containing 2% FBS, and 0.5% BSA). 293T cells were dissociated with TrypLE Express, washed with PBS containing 2% FBS, centrifuged at 1250rpm for 5 min and washed with cold FACS buffer. Cells were stained with the 7-AAD viability dye (eBiosciences) and viable cells were gated for the subsequent analysis. Finally, the cells were analyzed for GFP expression using a FACSCanto II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with the FACS Diva analysis software (BD Biosciences).

For HSCs, 1-$5x10^5$ cells were collected and resuspended in FACS buffer (PBS containing 2% FBS, and 0.5% BSA) to stain during 30 minutes at 4°C with CD34 PE-Cy7 (eBiosciences), CD133 PE (Miltenyi) and CD90 APC (eBiosciences). Then, cells were washed and resuspended again in the flow cytometry buffer containing 7-AAD as a viability marker.

For lymphocytes, 1-$5x10^4$ cells were collected and resuspended in PBS to stain during 30 minutes at 4°C with CD3-PE for T cells and CD19-APC for B cells. Then, cells were washed and resuspended in PBS.

Finally, all cells types for GFP expression were analyzed using a FACSCanto II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with the FACS Diva analysis software (BD Biosciences) or MACSQuant VYB (Milteny Biotech) and analysed with FlowJo (LLC).

7. Analysis of H2AX phosphorylation

K562 cells were nucleofected or transduced with the different nucleases (ZFNs or Cas9) and 48h later, 2.5 x 10^5 cells were collected and washed with cold FACS buffer (PBS containing 2% FBS, and 0.5% BSA). Cells were then fixed (80% methanol for 5 min), permeabilized (0,1% PBS-Tween for 20 min) and incubated with PBS+10% goat serum for 10 minutes to block non-specific protein-protein interaction, followed by the incubation with anti-histone γH2AX (phospho s139) antibody (1:100) (abcam, Cambrige, UK, ab81299) for 30 minutes at RT. Cells were washed with PBS and then incubated with goat-anti rabbit IgGH+L alexa fluor 647 antibody (1:10000) (abcam, Cambrige, UK, ab150077) for 30 minutes at RT. After washing with PBS, cells were analyzed for γH2AX expression using a FACSCanto II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with the FACS Diva analysis software (Becton Dickinson, Franklin Lakes, NJ). Isotype control antibody was rabbit IgG monoclonal (abcam) (0,1ug) also incubated 30 min at RT.

8. Quantification of nanoblades's Cas9 by ELISA.

The nanoblades containing the Cas9 and the Recombinant Cas9 (New England Biolabs, MA USA) were coated with the coating buffer containing 1% of Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) into 96 plates (Costar, Corning incorporated, NY, USA) and incubated overnight at 4°C. The samples were, then, diluted 1:100 in coating buffer. For the standard curve a serial dilution of recombinant Cas9 (New England Biolabs, MA USA) was diluted into the coating buffer.

24 h later, the plates were washed with PBS with 0.05% Tween20 and blocked with PBS+0.05%Tween20 +3%BSA (Sigma, Saint Louis, MO, USA). Then the plate was washed 4 times and incubated with the primary antibody; Cas9-7A9-3A3 (14697P cell signaling technology, mouse, diluted 1:1000 in PBS+3%BSA, and incubated at room temperature for 1 hour). After washing, the secondary antibody (goat anti-mouse HRP (F6009-X639F Southbiotech); diluted 1:10000 in PBS+3% BSA) was added. Finally, the mixed TMB substrate solution, containing HRP substrate was added and incubated during 20 minutes in the dark while shacking (Bethyl, Inc). Stop reaction was added in each well and the plate was read at absorbance 450nm in a Multiskan FC (Thermo Scientific).

9. Quantification of cleavage efficiency

Genomic DNA extraction from K562 cells was performed with QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the protocol for cells in suspension. The genomic region flanking the CRISPR and ZFNs target site for each treatment were PCR amplified using hWASP5Fw/hWASP5Rev primers (Table D). The 2.0kb fragment was purified using QiaQuick Spin Column (Qiagen, Hilden, Germany) following the manufacturer's protocols. 400 ng of total purified PCR products were mixed with 2µl 10X Taq DNA Polymerase PCR buffer and ultrapure water to a final volume of 20 µl, and subjected to a re-annealing process to enable heteroduplex formation (Figure 15B). After re-annealing, products were treated with Surveyor nuclease (Transgenomic, New Haven, CT, USA) following the manufacturer's recommended protocols and analyzed on 2% agarose gel. Two fragments of 1.2kb and 0.8kb will appear if the endonucleases have generated a DSB at the target site (Figure 15A and B).

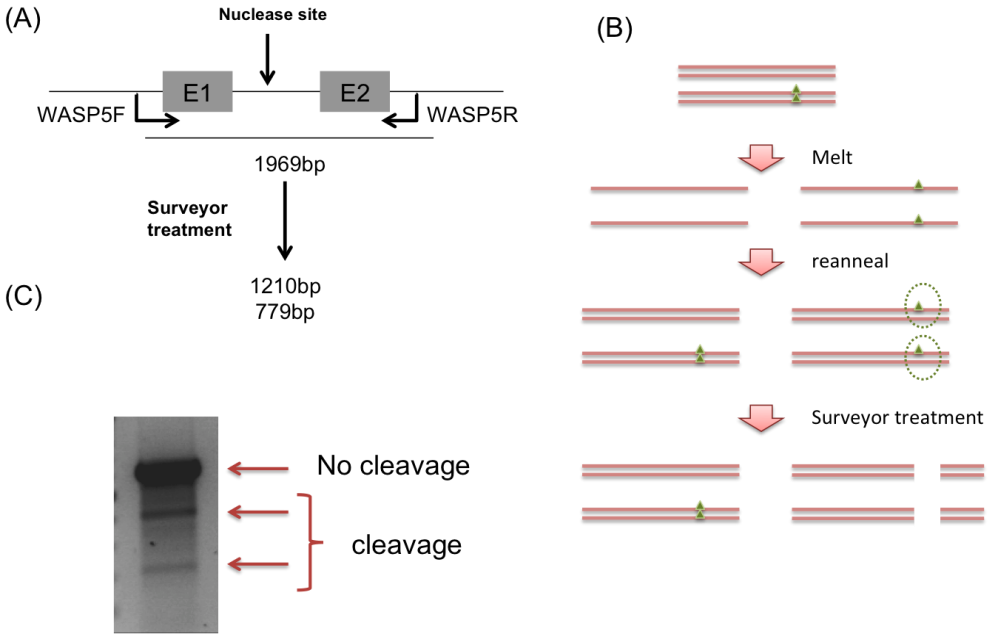


Figure 15. **A)** Scheme of the expected bands of the PCR for the WAS gene, including the primers used and the different expected bands after the treatment with Surveyor. **B**) Scheme of steps followed to do the surveyor treatment to detect mismatches. **C)** Representative image of surveyor nuclease assay of an experiment in which a treated population was assayed. The parental bands (1969) and resulting bands (1210 and 779) are shown.

The percentage of cleavage was determined by densitometry of the 2.0kb, the 1.2kb and the 0.8kb bands using the following formula: $indel\ (\%) = 100x\ (1 - \sqrt{(1 - fcut)}$ where $fcut = (b + c)/(a + b + c)$ where *a* is the integrated intensity of the undigested PCR product and *b* and *c* are the integrated intensities of each cleavage product [236].

Genomic DNA extraction for primary cells was carried out using the NucleoSpin tissue kit (Macherey-Nagel, GmBH &Co.) The genomic region flanking the cleavage made by the VLPs with two gRNA (301 and 305) was amplified by PCR with the hWASP2Fw/hWASP2Rev primers (Table D). The PCR product was run in a 1% agarose gel. When using the TPEM307/308alwas pair the band observed was of 351bp when there is no cleavage and two fragments of 351bp and 227bp are observed when the two gRNA cut the genome.

Primer's name	Sequence
hWASP5Fw	AAGCACTCACGATAGGCGTGG
hWASP5Rev	AAGTTCAGGTCAGGGGATTGT
hWASP2Fw	AGGGTTCCAATCTGATGGCG
hWASP2Rev	TTGAGAACTGGCTTGCAAGTCC
TPEM307Fvalwas	ATTGCGGAAGTTCCTCTTCTTACCCTG
TPEM308rvalwas	TTCCTGGGAAGGGTGGATTATGACGGG

Table D. Primers used for PCRs.

10. Off-target detection

Possible off-target sites for the CRISPR/Cas9_gRNA system were identified through a BLAST 41 and BOWTIE 42 algorithms using the entire sequence of the gRNA9 (GAGGCAGGAAGGACCAGGTC**TGG**) or just the first 16bp including the PAM sequence (GAAGGACCAGGTC**TGG**). For ZFNs we used the genome-wide tag scanner for nucleases off-sites 43 using the binding sites of both ZFNs as target.

We selected the 10 best potential off-targets for each nuclease (tables E and F) and analyzed the InDels generated by surveyor, as described previously, and using the primers pairs described in Table E and F. For gRNA 301 and gRNA305 we used the program CRISPRseek [237] and used the only 3 off target for gRNA 301 and the 4 of the most probable to give an off target for gRNA 305 (tables G and H).

Locus	Off- target sequence	Primers 5' → 3'	
Extended synaptotagmin-3	OT1: CAAGGACCAGGTCTGG	OT1 Fw	AGGCGGGCTGGGAAGCTTGA
		OT1 Rev	CCCGAAGCACCAGCCTCATG
Slit homolog 3 protein isoform 1 precursor	OT2: CAAGGACCAGGTCTGG	OT2 Fw	CAGGAGAGTGCTTGACAAAT
		OT2 Rev	AAGAGTCTTACGCCAAGAGG
Ankyrin repeat domiain-contaning protein 7	OT3: CAAGGACCAGGTCTGG	OT3 Fw	GAAACACTTCCAAATGAAGG
		OT3 Rev	ATCTGTCCCAGGCATTTATT
Voltage dependent calcium channel subunit alfa-2delat-2	OT4: GAAGGAGCAGGTCTGG	OT4 Fw	CCTTCTCTAGTTTGCCTAGG
		OT4 Rev	TATGGACTCCAGGAGTACAG
Armadillo repeat-containing protein 12 isoform a-b	OT5: GAAGGAGCAGGTCTGG	OT5 Fw	GCAGATCACATTGGACCGTA
		OT5 Rev	GGCCAAACCCAATAGACAAT
Uncharacterized protein LOC100505478	OT6: GAAGGAGCAGGTCTGG	OT6 Fw	CTCCTGGCACTTACTGTCTA
		OT6 Rev	TTCCGTGATCTCACCTAAGC
Septin-9 isoform a	OT7: GAAGGAGCAGGTCTGG	OT7 Fw	GGTTTGGATGCTGAGGGTGA
		OT7 Rev	CACCCAGTTTCGACAGTTGT
Small G protein signalingmodulator 3 isoform 1	OT8: GCAGGAAGGACCAGGGCTGG	OT8 Fw	GAGGAGCTGCTCTACCGGGTAA
		OT8 Rev	ACCTTAGCCACCCCGCGAACG

Table E. Potential off-target sites for CRISPR/Cas9_gRNA9. The table show the different locus potentially targeted by the gRNA9 (left), the off-target sequence (middle) and the primers pair used to perform the PCR for the Surveyor analysis (right).

Locus	Off- target sequence	Primers 5' → 3'	
WD repeat domian 33	CCTTGGGGCCCA*TGCAT*GTCCTGAGGCCG	OT1 Fw	AGGGTGAGGTCCTCTCATCTC
		OT1 Rev	TGAACATGGCTCAAATGGGG
Tumor necrosis factor receptor	CCTTTGGCCCCA*TTCCT*TGGGCCCAAATA	OT2 Fw	CCCTAGAGAGTTGCCTGACC
		OT2 Rev	GGAGGCTGAAGAATGATTGC
40S ribosomal protein S4	CCTTTAGGGCCA*AGTTC*TGGACCCAAAGG	OT3 Fw	TTGCCTACTGTAGCACCACAGC
		OT3 Rev	AAGCACTTCAAGGGACAATTAGG
Uncharacterized protein C9orf106	CAGCCTCATGACAAGAGGAGATGAGGCAG	OT4 Fw	TGTTCGGCTCATCATTCCATC
		OT4 Rev	TGTGTCTGTAGTCCCAACTAC
Spectrin beta chain	CAGCTTCATGAC*CCCCT*GTCATGAGGAAG	OT5 Fw	AGCATGCATGCTGTGTGTTCTG
		OT5 Rev	TGGAGTACATTCCAGCAGGACC
Mitochondrial uncoupling protein 2	CTACCTCATGAC*TCACCT*GTCATGAGGTTG	OT6 Fw	AAATTAGCCAGTGTGCTGGTGC
		OT6 Rev	TGGCTCGCCCACAGATGCCAGC
IRRE-like protein 1 isoform 1	GCTGTGGGCCCA*TGGGT*GTCATGCAGCAG	OT7 Fw	AGAGGCGCTTGATGGAGGAG
		OT7 Rev	AGTGATTCTCCTGCGTCAGC
Zinc finger CW-TypePWWP domain	CCTCTGGGCCC*TACACA*GACATGAGTCAG	OT8 Fw	TTATGCTTAAGGCCTTCAACTGAC
		OT8 Rev	ATGGCCTACTACACAGTATATAC

Table F. Potential off-target sites for ZFNs. The table shows the different locus potentially targeted by the ZFNs (left), the off-target sequence (middle) and the primers pair used to perform the PCR for the Surveyor analysis (right).

Locus	Off- target sequence	Primers 5' → 3'	
Intronic sequence Chromosome 1	CAAGAAATTCAAAGAGTCGCCAG	OT-1 Fw	GTAAATGTTCAATAACTATT
		OT-1 Rev	TTATTTAGAGACAGGGTCTC
FGF14 Chromosome 13	CAAGCATCCCAAAGAGTGGCTGG	OT-2 Fw	CATTTACTTTAGAAGAGTTC
		OT-2 Rev	CCTTGACATATACAGTCCTC
Intronic sequence Chromosome 12	AAAGCATCCCAAAGAGTCTCAAG	OT-3 Fw	ATTAGCCAGGCATGGTAGCG
		OT-3 Rev	AAGTAGGTCAGTGGGAAGCT

Table G. Potential off-target sites for CRISPR/Cas9_gRNA 301

Locus	Off- target sequence	Primers 5' → 3'	
LINC00607 Chromosome 2	CACGCTTAGACGAAAATGCTGGG	OT-4 Fw	TGCAGTTACTCATTCTCAGA
		OT-4 Rev	GGTATGGAGGCAAGTGTGGA
KIAA1217 Chromosome 10	AATGCTTGGAAGAAAATGCAGGG	OT-10 Fw	TTCAACAACAGTGCCAAGAT
		OT-10Rev	TCCCTTATCAGGTGTACGAT
Intronic sequence Chromosome 4	CATGCTTGCACGAAAATGATGGG	OT-12 Fw	TAGAGGTTATTGCTGCCCTT
		OT-12Rev	ACCACAACCATCCAATTACC
Intronic sequence Chromosome 2	GATGCTTGGGAGAAAAAGCTTGG	OT-14 Fw	CCTATGTATATTTGAAATT
		OT-14Rev	GTGTTATATCTGTTATGTGC

Table H. Potential off-target sites for CRISPR/Cas9_gRNA 305

11. Efficiency and fidelity of HD-donor insertion into the *WAS* locus.

K562 cells were nucleofected with the plasmids expressing the different endonucleases (pSZFN24/pSZFN55; pSZFN24EL/pSZFN55KK or pSCas9WP/SELg9) and the donor DNA expressing eGFP and NeoR genes (Figure 16). Cells transfected with the nucleases and the donor DNA were selected with neomycin for 20 days and GFP positive cells sorted by FACS Aria II (BD Biosciences) and seeded into a 96well plate to obtain single cells (1 cell/5 wells).

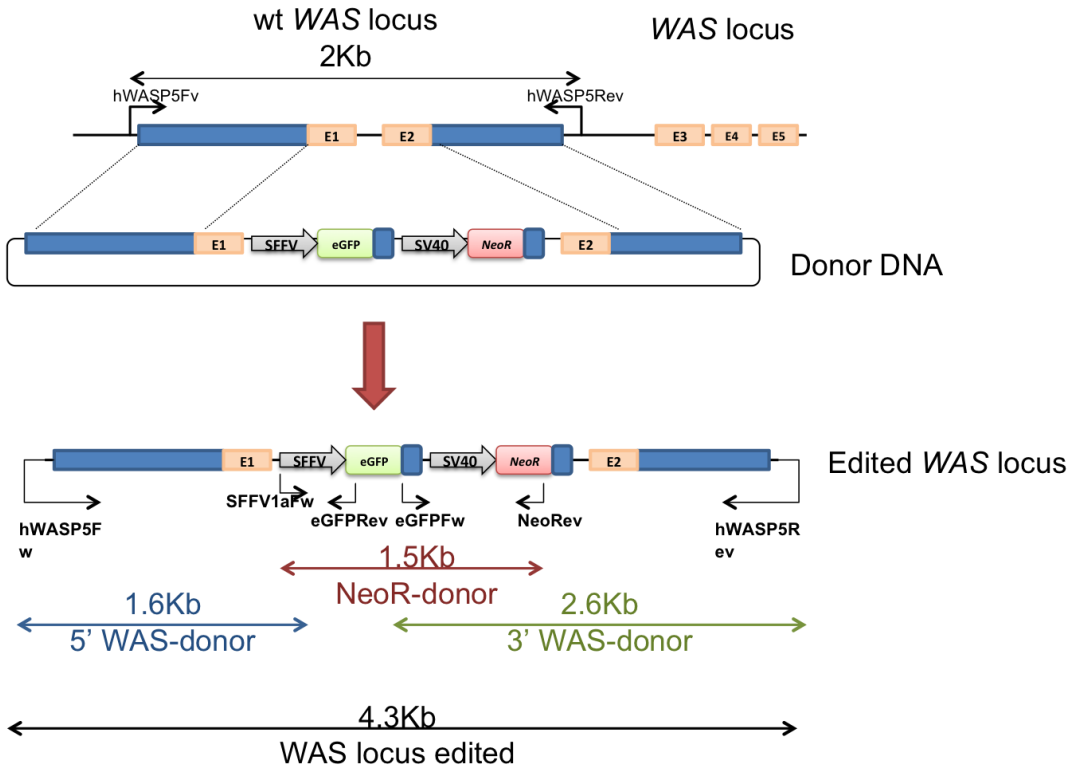


Figure 16. Scheme showing the first 5 exons of the WAS locus (E1-E5) and the target site for ZFNs and CRISPR (top). The middle diagram shows the donor DNA used to edit the WAS locus containing the eGFP (Green), the NeoR (Red) expression cassettes and the homology arms in 5' and 3' (in blue). The lower diagram shows the expected outcome of the edited WAS locus after homologous recombination with the donor DNA. The different primers are shown as black arrows, and expected size of the different PCR products are shown with arrows of different colors. (Modified from Gutierrez *et al*. 2017)

Clones were grown in the presence of neomycin and genomic DNA extracted from over 50 clones from each condition. The DNAs were used as templates for PCRs using 1- a primer pair (hWASP5Fw/hWASP5Rev) flanking the WAS target sequence, outside the donor homology domains, 2- two primers pairs that amplify the endogenous *WAS* and the donor DNA at the 5′ (hWASP5Fw/eGFPRev) (Figure 16) and at the 3′ (eGFPFw/hWASP5Rev) (in green in figure 16) junctions and 3- a primer pair (SFFV2aFw/NeoRRev) (in red in figure 16) that amplify an internal fragment from the donor DNA (Table G). All the PCR were carried out using the Kappa taq polymerase (Kappa biosystem).

Primer's name	Sequences
eGFPRev	CGTTTACGTCGCCGTCCAGC
eGFPFw	GACGTAAACGGCCACAAGTTCA
SFFV2aFw	CTTCTGCTTCCCGAGCTCTA
NeoRev	AGTGACAACGTCGAGCACAG

Table I. Specific primers for HR verification.

12. HS4 and synthetic insulator design and synthesis

We constructed two different HS4 based insulators: - the cHS4 core (a 250-bp fragment from the 5' of the HS4 element) and - the HS4Ext (a 400-bp fragment containing the cHS4 core plus 150 bp of the 3´flanking sequences. All sequences were synthesized by GenSscript (GenScript USA Inc. NJ, U.S.A). Plasmid SFFV-GFP (SE) containing the different HS4 elements within LTR were constructed by subcloning the LTR fragment into an *XbaI* site of pUC19 (New England Biolabs, Ipswich, MA, USA). The different HS4 elements were cloned into the unique *Bbs1* site of the subcloned LTR to obtain LTR-HS4core and LTR-HS4Ext. The final SE-HS4core and SE-HS4Ext lentiviral plasmids were obtained by replacing the LTR from the SE plasmid with the LTR-HS4core and LTR-HS4Ext, respectively, and using the XbaI restriction enzyme and standard molecular biology techniques. All the constructs generated were verified by automated DNA sequence analysis and with multiple restriction enzyme digestions.

RESULTS

“Results! Why, man, I have gotten lots of results. I know several thousand things that wouldn't work.”

Thomas A. Edison

Results

1. Construction of *WAS*-specific ZFNs and CRISPR/Cas9 systems.

WAS-specific homodimeric ZFNs (ZFNs_wt) and heterodimeric ZFNs (ZFNs_mt), targeting the first intron of the *WAS* gene, were designed by Sangamo as described in material and methods. To be able to compare ZFNs to CRISPR/Cas9 system we designed and improved the Cas9 nuclease based on previous publications (described material and methods) and searched for gRNA at the same location as the ZFNs (Figure 17). We selected all possible gRNAs in a 100 bp region 5' and 3' from the ZFNs cutting site and analyzed for potential off-targets sites using BLAST analysis. Only one gRNA (gRNA9: 5′-gaggcaggaaggaccaggtc-3′) compiled with the required properties: 1- Targeting the same region as the ZFNs and 2- having no homology with other regions in the genome.

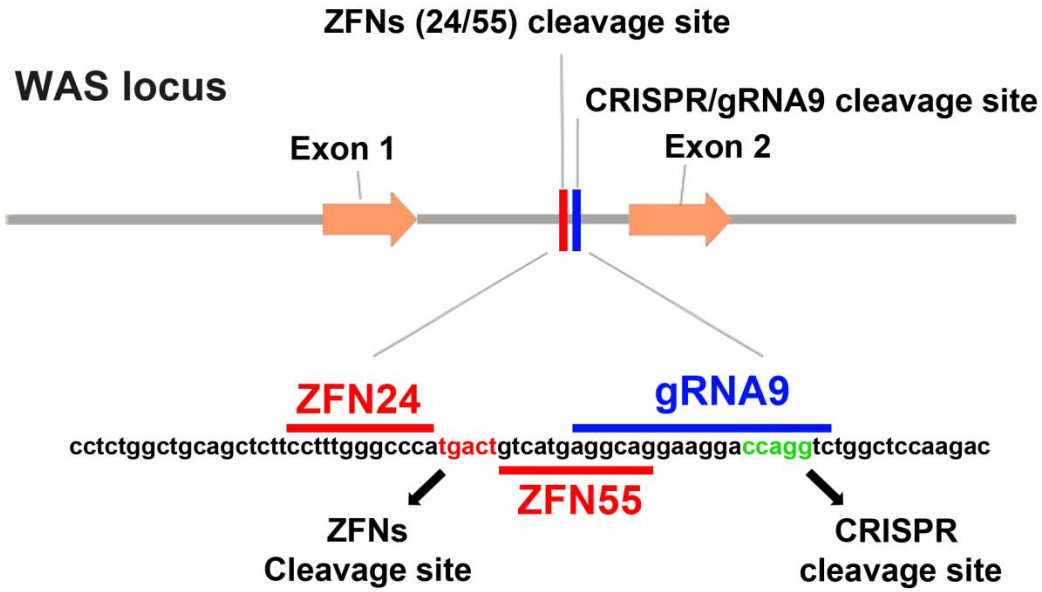


Figure 17. Diagram of a fragment of the WAS locus showing the exon 1 and exon 2 (red arrows), the target sites of the *WAS*-specific ZFNs and the CRISPR/Cas9_gRNA9.

As the efficiency and safety of different specific nucleases can vary depending on expression levels and this can be highly influenced by the delivery system used, we constructed different plasmids that can be used to deliver the nucleases by plasmid nucleofection and also by IDLVs. We therefore cloned the optimized Cas9 (optCas9) and the gRNA9 into lentiviral vector plasmids as described in materials and methods to obtain the SCas9 and the SELgRNA9.

2. Cutting efficiency of WAS-specific CRISPR and ZFNs.

Once we had the different endonucleases cloned into lentiviral plasmids, we transfected them either by nucleofection or by integrative-deficient lentiviral particles (IDLVs) into the target cells:

a. Efficacy of the CRISPR system in K562 cells using plasmid nucleofection.

In order to test our designed CRISPR/Cas9 system, we nucleofected K562 cells with the SCas9 and the SELgRNA9 plasmids (Figure 18 A and B). Next, we extracted genomic DNA and proceeded to do a PCR of the *targeted locus* with the WASP5F and WASP5R primers (table D in material and methods and Figure 15A), performed the surveyor essay and visualized the bands in a 2% agarose gel (Figure 18C). We observed that our CRISPR/Cas9 system was able to disrupt the *WAS* gene in up to 13% of the K562 cells only if, as expected, both plasmids, the SELgRNA9 and the SCas9, were nucleofected together (Figure 18C).

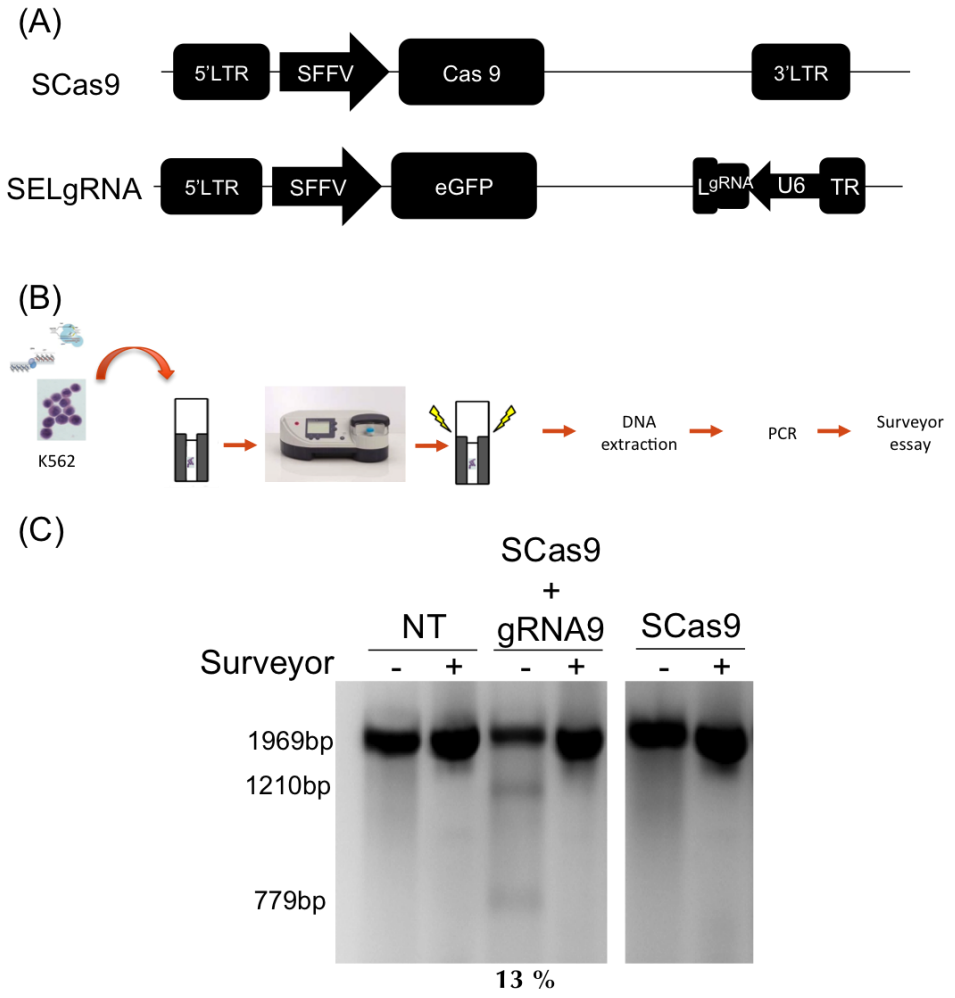


Figure 18. The CRISPR/Cas9 system allows efficient targeting of the *WAS* locus in K562 cells. **A)** Scheme of the LV plasmids expressing the optimized Cas9 under the SFFV promoter (SCas9) (SC9WP- top) and the gRNA driven by the U6 promoter in (SELgRNA9- bottom). **B)** Scheme of the procedure to deliver the plasmids into K562 cells by nucleofection. **C)** 2% Agarose gel showing the bands obtained after PCR of the intron region (WASP5F and WASP5R primers) using control K562 cells NT (left), cells nucleofected with the SCas9 plasmid (SCas9) and cells nucleofected with the Scas9 and SELgRNA9 plasmids (Cas9 + gRNA9) with (+) or without (-) treatment with Surveyor.

b. Efficacy of WAS-specific ZFNs in K562 cells using plasmid nucleofection.

As for the CRISPR/Cas9 system, the ZFNs, both homodimeric, ZFN24 and ZFN55 (ZFNs_wt) and heterodimeric, ZFN24EL and ZFN55KK (ZFNs_mt) were cloned into a lentiviral vector backbone harboring the SFFV promoter (Figure 19A) and we analyzed their cutting efficiency by nucleofection of K562 cells (Figure 19B). We observed that the efficiency for both nucleases was very similar, around 27%.

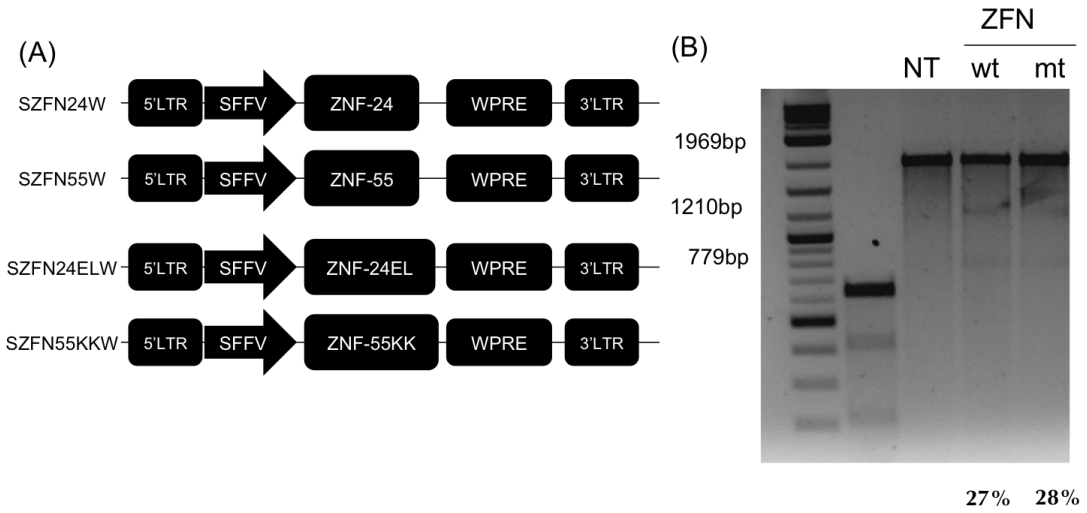


Figure 19. **A)** Scheme of the different ZFNs construct under the SFFV promoter and a LV backbone. ZFN24 and ZFN55 are the two homodimeric arms of the ZFNs_wt (two top) while ZFNs-24EL and 55-KK are the two heterodimeric arms of the ZFNs_mt (two bottom). **B)** Electrophoresis gel of the surveyor essay of nucleofected K562 cell. First lane shows the surveyor control (blank) , second lane shows cells nucleofected with ZFNs_wt and third lane shows cells nucleofected cells with the ZFNs_mt. Percentage of cleavage is shown at the bottom of each lane.

c. Efficacy of the different endonucleases system using IDLVs

IDLVs, as a non-integrating delivery system, are an interesting alternative to plasmid delivery because we can adapt their tropism and thus, entry into specific target cells. Moreover, they have the ability to incorporate different elements (endonucleases, donor DNA templates, iRNA). Finally, they have the potential to be used *in vivo*. We, therefore, generated IDLV particles expressing each of the CRISPR components; SCas9 and SELgRNA9 and each of the ZFNs_mt using the plasmids showed in Figures 18A and 19A. Table J shows the average number of particles per ml obtained with the different IDLVs.

Vector	PN/ml
SZFN24W	$2x10^9 \pm 1x10^9$
SZFN55W	$1,61x10^9 \pm 1,32x10^9$
SZFN24ELW	$1,89x10^{10} \pm 7x10^9$
SZFN55KKW	$1,87x10^{10} \pm 9,7x10^9$
SCas9W	$9,03x10^9 \pm 1,40x10^{10}$
SELg9W	$8,72x10^9 \pm 7,32x10^9$

Table J. Average of the number of particles (PC) per ml of the different IDLVs vector and the different endonucleases ± SD (Concentrated 100times)

We first compared the efficiency of not concentrated IDLVs expressing the heterodimeric (ZFNs_mt) versus the homodimeric (ZFNs_wt) ZFNs. K562 cells were transduced using a PNC (particle number per cell) = 40 of each IDLV pair (SZFN24W/SZFN55W or SZFN24ELW/SZFN55KKW) and the cutting efficiencies determined by surveyor assay. The data showed that both IDLVs were able to cut efficiently the *WAS* locus.

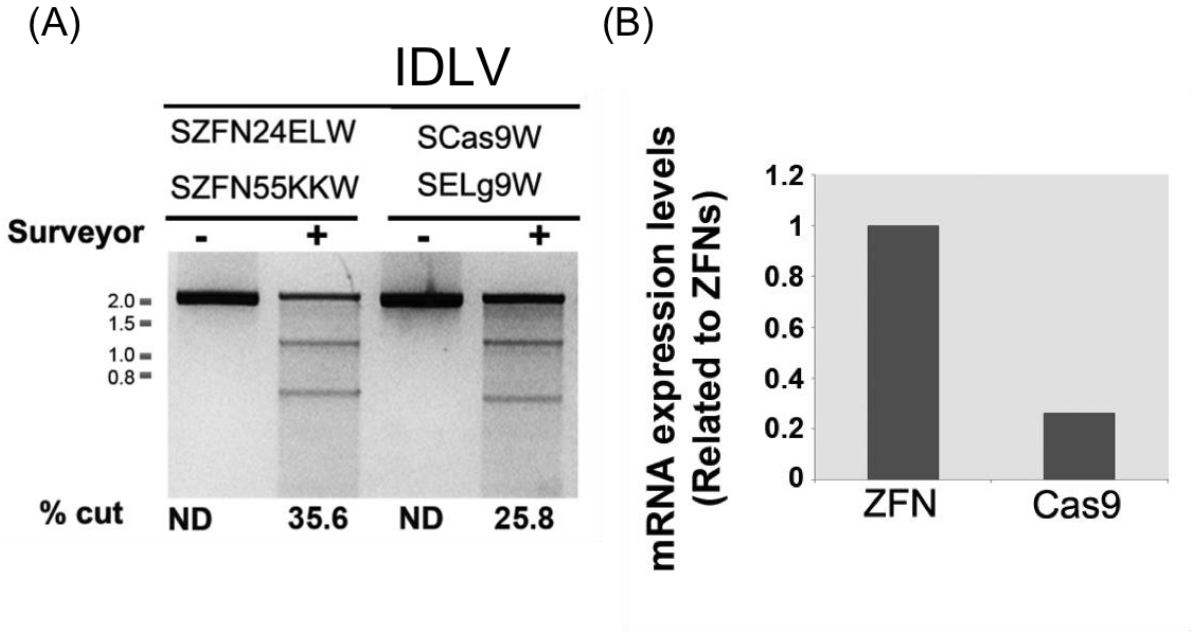


Figure 20. Comparative analysis of dual IDLVs CRISPR and ZFNs systems to edit the *WAS* locus. A) Surveyor analysis of K562 cells transduced with IDLVs expressing ZFN24EL and ZFN55KK (Left lines) or Cas9 and gRNA9 (Right lines) at a PNC= 200 each. Nuclease activity at the target site was monitored by PCR and digestion with the Surveyor nuclease (+) and detection by agarose gel electrophoresis. The activity is shown as percentage of cleavage (estimated by densitometry of the bands) and indicated under each line. **B)** mRNA quantification of K562 cells transduced with SZFN24ELW/SZFN55KKW (ZFNs) or with Scas9W/SELg9W (Cas9) IDLVs. The total amount of mRNA expressing the nucleases, ZFNs or Cas9, was performed as described in material and methods using common primers to the WPRE region.

We next compared the ZFNs_mt with the CRISPR system to see whether they reached similar efficacies. K562 cells were transduced using a PNC= 200 of each IDLV pair (SZFN24ELW/SZFN55KKW or SCas9/SELgRNA9) and the cutting efficiencies were determined (Figure 20A). Our data showed higher cutting efficacies of IDLVs-ZFNs_mt compared to the dual IDLV-CRISPR system (35.6% or ZFNs versus 25.8% for CRISPR). The analysis of the mRNA expression levels of both systems using primers directed to the WPRE (present in all constructs) showed that the CRISPR/Cas9 IDLVs expressed lower RNA levels compared to the ZFNs (Figure 20B). These differences in RNA expression could be responsible of the lower cutting efficacy of the CRISPR/Cas9 systems.

i. Optimization for the delivery of CRISPR/Cas9 system as IDLVs.

In order to compare specificity of ZFNs and CRISPR, it is important to be able to achieve similar cutting efficiencies, so, we decided to improve the CRIPSR system by: 1- expressing both the gRNA and Cas9 in the same lentiviral plasmid 2- Introducing different modifications that could improve expression levels of the Cas9 or the gRNA (Figure 21A). We inserted the U6-gRNA9 expression cassette in three different positions; LTR (SCas9Lg9W), 3' of the Cas9 (SCas9Xg9W), and 5' of the Cas9 (SCas9Eg9W) and also analyzed whether the presence of the Woodcuck hepatitis virus Posttranscriptional Regulatory Element (WPRE) improved the system (SCas9Lg9 versus SCas9Lg9W).

Vector	PN/ml
Eg9SC9W	$4{,}31x10^{9} \pm 4{,}50x10^{9}$
SC9Xg9W	$3{,}33x10^{10} \pm 1{,}26x10^{10}$
SC9Lg9W	$3{,}74x10^{10} \pm 7{,}93x10^{9}$

Table K. Average of the number of particles (PN) per ml of the different all-in-one IDLVs vector ± SD (Concentrated 100times).

We obtained IDLVs from the different constructs (Table K) and analyzed their efficacy in K562 cells using a PNC of around 300. As expected the WPRE sequence enhanced the efficacy of the IDLVs (30.6% for SCas9Lg9 versus 35.5% for the SCas9Lg9W IDLVs) (Figure 21B).

We observed a similar percentage of cutting efficiency in all of them, although the vector harboring the gRNA in the LTR and including the WPRE (SC9Lg9W) showed the higher cutting efficiency. We therefore decided to continue our further studies with this construct.

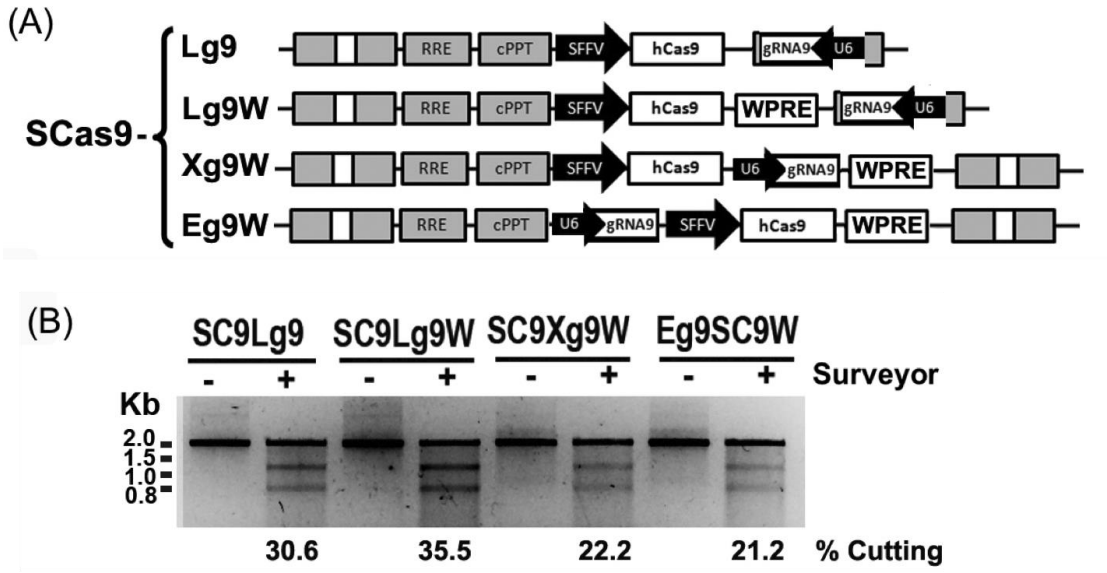


Figure 21. **Optimization of the CRISPR/Cas9 delivery system. A)** Drawing of the different all-in-one lentiviral plasmids constructs expressing human codon-optimized Cas9 gene through the SFFV promoter and the gRNA9 through the U6 promoter. The U6-gRNA9 expression cassette was inserted inside the 3'LTR (SC9Lg9 and SC9Lg9W), just 3' the Cas9 gene (SC9Xg9W) or just 5' the Cas9 gene (SC9Eg9W). The WPRE element was inserted in all constructs with the exception of the SC9Lg9 (top) for comparison with the SC9Lg9W. **B)** Analysis of cutting efficiency of all-in-one IDLVs represented in A. All of the IDLVs were used with equal PNC (300). Nuclease activity at the target site was monitored by PCR (-), digestion with the Surveyor nuclease (+) and detection by agarose gel electrophoresis. The activity is shown as percentage of cleavage (estimated by densitometry of the bands) and indicated under each line.

3. Specificity of WAS-specific nucleases.

Once we had improved the CRISPR system that could render similar efficacies than ZFNs we decided to compare the specificity of the homodimeric ZFNs_wt pair (SZFN24W, SZFN55W), the heterodimeric ZFNs_mt pair (SZFN24ELW and SZFN55KKW) and CRISPR/Cas9 (SC9Lg9) using both delivery methods: Plasmid nucleofection and IDLVs.

a. Comparison of the specificity of endonucleases delivered by plasmid nucleofection.

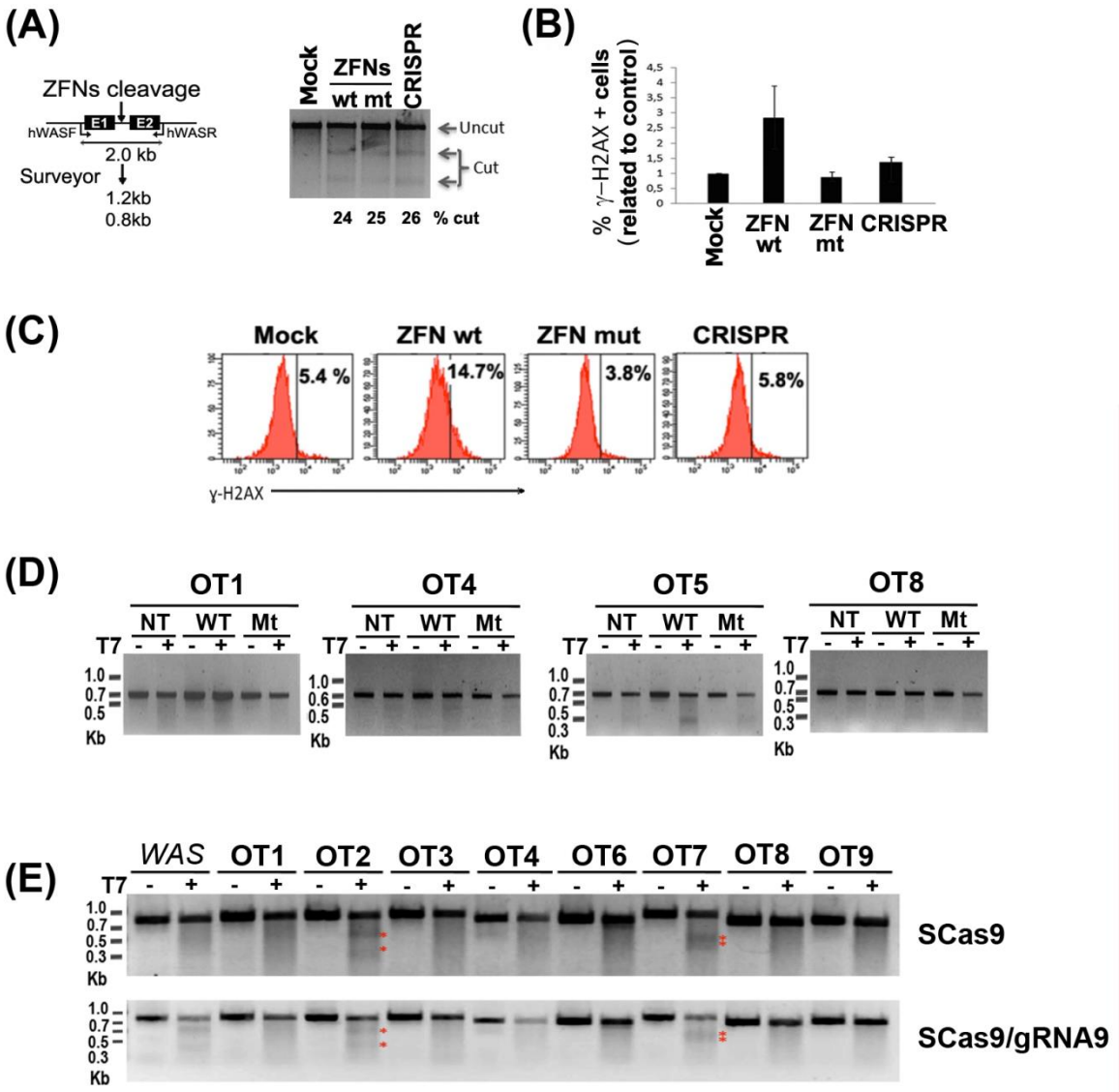


Figure 22. Comparison of efficiency and specificity of *WAS*-specific zinc finger nucleases and CRISPR system delivered by nucleofection. A) Analysis of the cutting efficiency of the different specific nucleases. Nuclease activity at the target site was monitored by PCR, digestion with the Surveyor nuclease (left) and detection by agarose gel electrophoresis (right). The activity is shown as percentage of cleavage (estimated by densitometry of the bands) and indicated under each line. **B)** Graph showing increment in double strand breaks (γ-H2AX positive cells) of K562 cells transfected with the different plasmid expressing ZFNs_wt, ZFNs_mut or CRISPR related to Mock K562. Average of at least two separate experiments. **C)** Representative histograms for K562 cells nucleofected with irrelevant plasmid (Mock), homodimeric ZFNs (ZFNs_wt pair), heterodimeric ZFNs (ZFNs_mut pair) or CRISPR-gRNA9 (Cas9 and gRNA9) stained with antibodies against phosphorylated γ-H2AX to detect double strand breaks. The percentage of cells positives for phosphorylated γ-H2AX is indicated in each histogram. **D)** Analysis of indel generation in K562 cells with ZFNs nucleofection of different off target sites (OT1, 4, 5, 8) of no transduced cells and cells nucleofected with ZFNs_wt pair and ZFNs_mt pair. **E)** Analysis of index generation for the different off targets sites (OT1,2,3,4,6,7,8,9) for nucleofected K562 with SCas9 alone and SC9/gRNA using Surveyor assay (-) without the endonucleases and (+) with the endonuclease. The red asterisk shows unspecific bands with and without gRNA.

In order to compare specificity, we required populations that had similar cutting efficiencies with the different endonucleases. This was easily achieved by plasmid nucleofection since we observed around 20% efficacy in all systems (Figure 22A). To evaluate the unspecific DSB generation (off-targets) we stained the cells for γH2AX (Figure 22B and C).

The H2AX is a variant of the H2A family that is phosphorylated (γH2AX) as the first step in recruiting DNA repair proteins upon DSBs. The repair processes generate foci that can be visualized by staining for γH2AX. These foci represent the DSBs in a 1:1 ratio and are used as biomarkers for DNA damage [238]. The differences observed in γH2AX staining on cells edited at similar efficiencies in the *WAS* locus will represent therefore differences in off-target activity. As can be observed in Figures 22B and 22C, γH2AX staining is similar between SC9Lg9W, the ZFNs_mt pair and the mock (nucleofected with irrelevant plasmid), indicating similar off-target activity of these two *WAS*-specific nucleases. Moreover, since we could not observe a γH2AX increment (related with the mock), we can say that the off-target activity of these two nucleases is below the limit of detection of this technique. However, the ZFNs_wt pair presented higher γH2AX staining compared to control and thus corroborate the potential of these technique to detect off-target activity as well as the higher off-target activity of ZFNs_wt compared to ZFNs_mt and our CRIPSR system.

We next analyzed the potential of off-target sites by in-silico analysis. We used the genome-wide tag scanner for nucleases off sites [239] for the ZFNs and BLAST [240] and Bowtie [241] for CRISPR/Cas9. We selected the 8 highest scoring off-targets for CRISPR/gRNA9 (Table E) and ZFNs (Table F)) and analyzed InDel generation using the surveyor assay, by amplifying the genomic DNA with the different primers directed against the off-target sequences followed by the surveyor assay and electrophoresis gel to see the cleavage. Only 4 and 7 off-target are shown for ZFNs and CRISPR respectively. However, no detectable off-target activity was observed when using this technology; most probably because the limit of detection of the surveyor assay is around 2% (Figures 22D and 22E for ZFNs and CRISPR respectively).

b. Comparison of the specificity of endonucleases delivered by IDLVs

We next generated SCas9Lg9W IDLVs particles as well as IDLVs expressing each ZFNs and compared their specificity to disrupt the *WAS* locus. K562 cells were co-transduced with IDLV-ZFN24W/IDLV-ZFN55W (IDLV- ZFNs_wt), IDLV-ZFN24ELW/IDLV-ZFN55KKW (IDLV-ZFNs_mt) or with the all-in-one SCas9Lg9W IDLV at different PNC in order to reach similar cutting efficiencies. 48 hours later we analyzed DSBs by γH2AX and InDels generation at the target site (*WAS* locus) by surveyor (Figure 23).

It is important to take into account that in order to be able to compare the different nucleases in the target cells, these have to be edited at similar levels, as higher cutting efficiencies could be translated into higher off-target activities. We therefore selected the K562 cells edited between 19 to 25% to perform a comparative analysis (Figure 23).

We required 2-9 times higher PNC of the IDLV-CRISPR than IDLV-ZFNs in order to reach similar efficiencies (Figures 23 and 24A). Interestingly, as observed with nucleofection, we observed similar levels of γH2AX positive cells in CRISPR- and ZFNs_mt- treated K562 cells when edited at similar levels (Figures 23 and 24B and C).

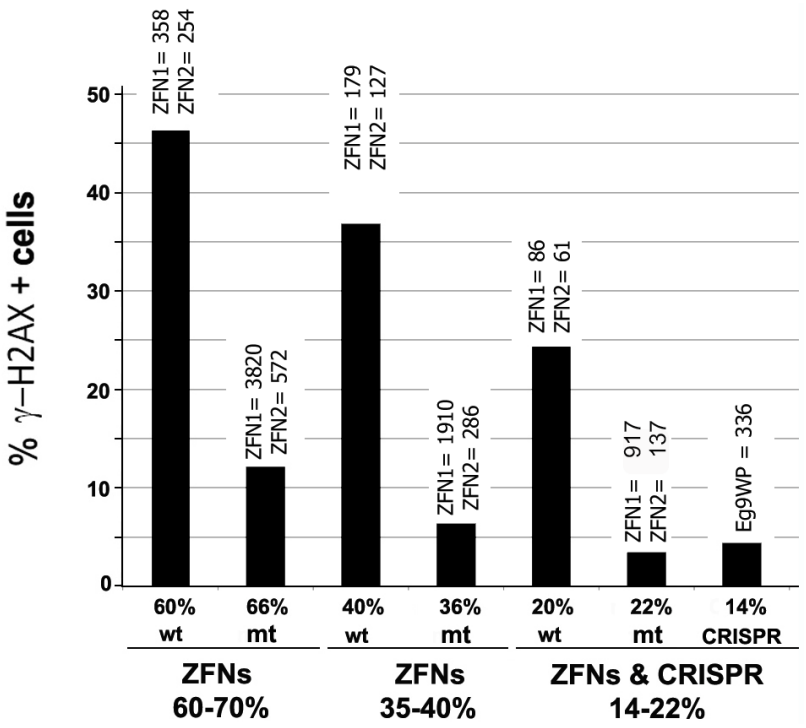


Figure 23. Comparison of the specificity of *WAS*-specific zinc finger nucleases and CRISPR system delivered by IDLVs at different PNC. Graph showing the percentage of cells stained positive for γ-H2AX (indicative of DSB) after transduction with different IDLVs expressing each homodimeric ZFNs (ZFN24 and ZFN55), heterodimeric ZFNs (ZFN24EL and ZFN55KK) or a unique IDLV expressing Cas9 and the gRNA9 (CRISPR). K562 cells were transduced with different volumes of each concentrated IDLVs. For ZFNs-IDLVs we used 50µl, 25µl and 12µl (achieving 60-70%, 35-40% and 20-22% cutting efficiency respectively) and for SC9Lg9W IDLVs we used 150µl to obtain 14% cleavage. The number of particles per cell was estimated by RT-PCR and is indicated at the top of each bar. The different samples were stained with antibodies against γ-H2AX and analyzed by flow cytometry to determine the % of γ-H2AX positive cells related to an isotype control. Nuclease activity at the *WAS* site at the different samples was monitored by Surveyor nuclease and indicated at the bottom of each bar.

However, cells edited at similar levels with ZFNs_wt showed always higher γH2AX staining compared to ZFNs_mt or CRISPR, indicating higher off-target activity (Figure 23 and 24B and C).

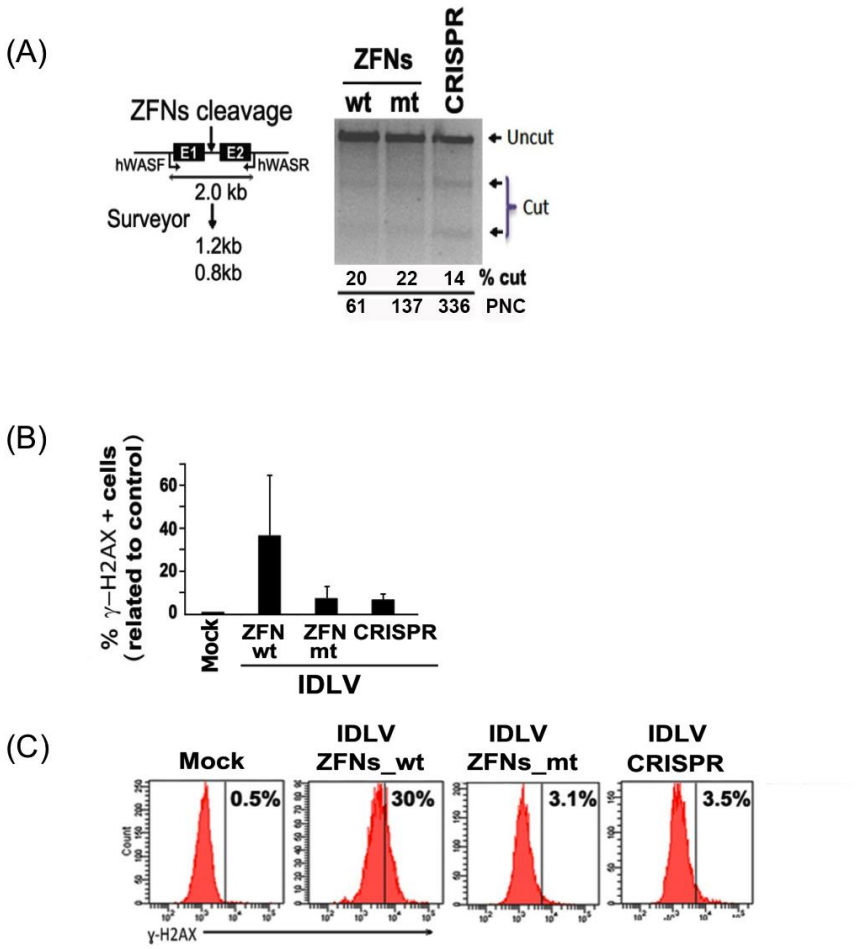


Figure 24. Comparison of efficiency and specificity of *WAS*-specific zinc finger nucleases and CRISPR system delivered by IDLVs. A) Analysis of the cutting efficiency of the different specific nucleases using different PNCS (61 and 137 for ZFNs_wt and mt respectively and 336 for CRISPR) to reach 19-25%. Nuclease activity at the target site was monitored by PCR and digestion with the Surveyor nuclease (left) and detection by agarose gel electrophoresis (right). The activity is shown as percentage of cleavage (estimated by densitometry of the bands) and indicated under each line. **B)** Graph showing increment in DSB (γ-H2AX positive cells) related to untreated K562 cells or transduced with the different IDLVs combinations reaching 15-20% cutting efficiency. Average of at least two separate experiments. **C)** Representative histograms for human control K562 cells (NT), K562 cells transduced with IDLVs expressing each IDLV-ZFNs_wt pair, each IDLV-ZFNs_mt pair or a unique IDLV expressing Cas9 and the gRNA9 (IDLV-CRISPR) stained with antibodies against γ-H2AX to detect double strand breaks. The percentage of cells positives for γ-H2AX is indicated in each histogram.

4. Efficacy and specificity of ZFNs and CRISPR/Cas9 for homology direct genome editing of *WAS* locus in K562 cells.

Above, we evaluated the endonuclease activity of the ZFNs and CRISPR/Cas9 for gene disruption of the *WAS* locus by NHEJ. In a next step, we aimed to investigate the efficiency and specificity of *WAS*-specific ZFNs_wt, ZFNs_mt and CRISPR systems for homology direct genome editing of *WAS* locus.

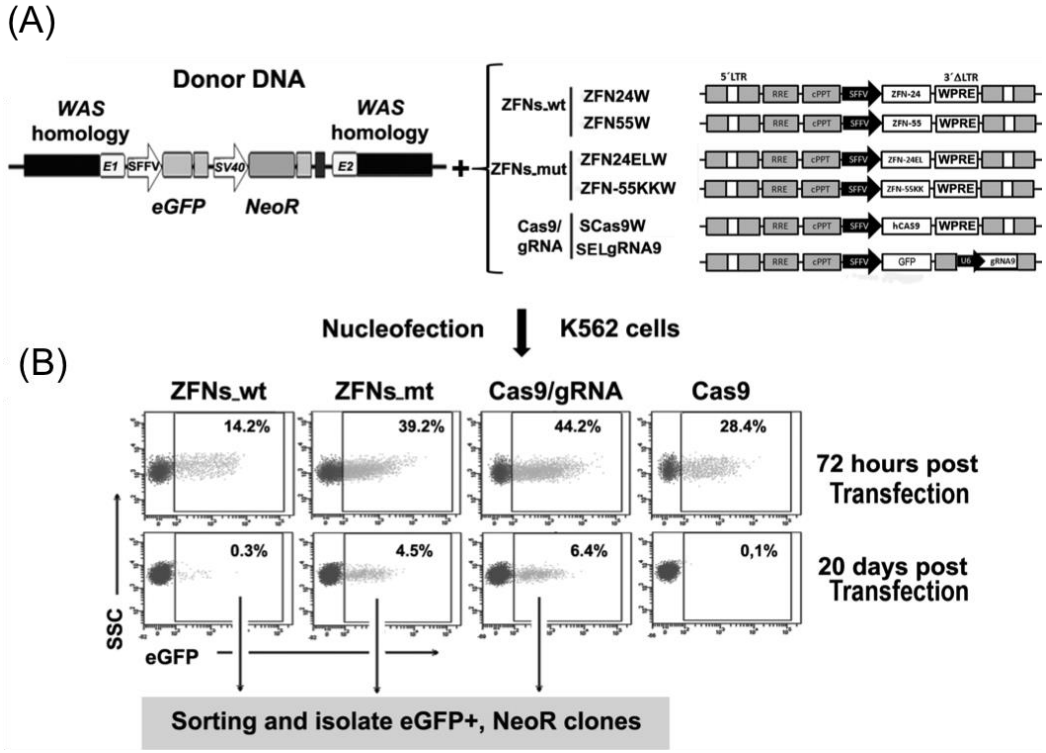
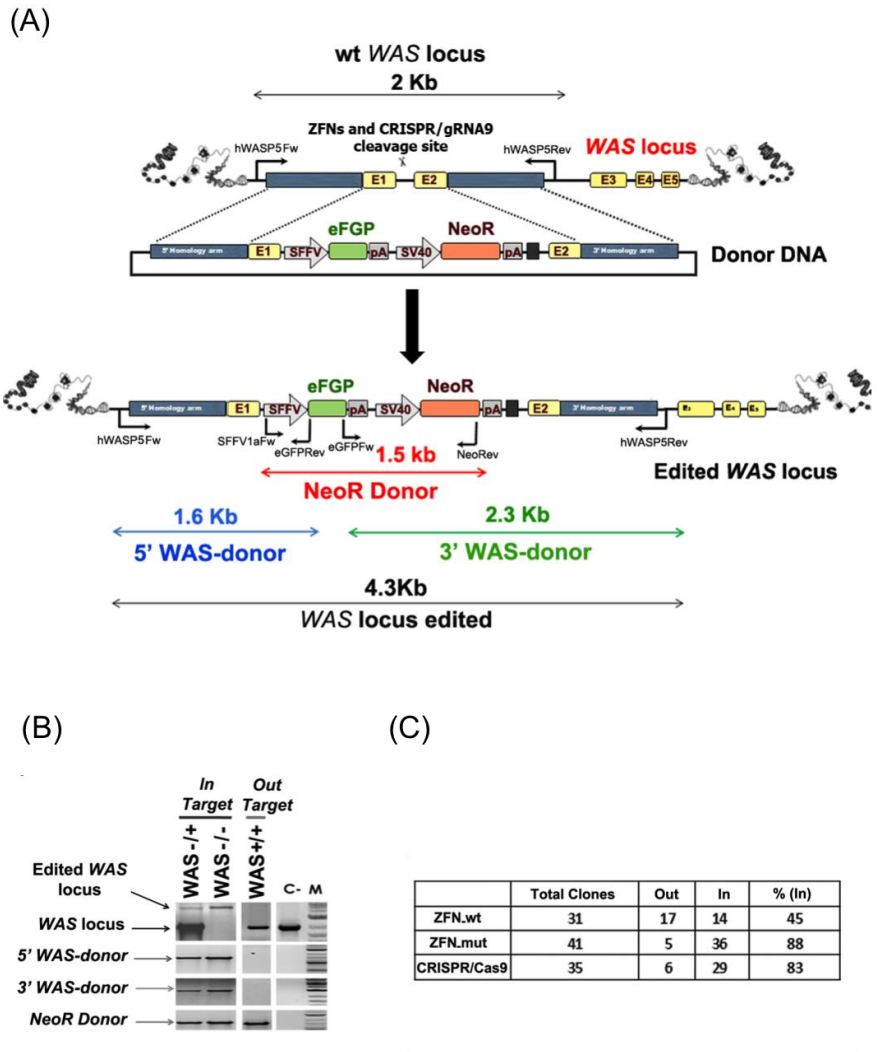


Figure 25. Analysis of the efficiency and safety of ZFNs and CRISPR/Cas9 for HR-directed WAS genome editing. A) Scheme showing the procedure to insert a donor DNA (harbouring eGFP and NeoR expression cassettes) into the *WAS* locus using different specific nucleases. ***B)*** Plots indicating the GFP percentage after 72 hours (top graphs) and 20 days (bottom graphs) after nucleofection with donor DNA and ZFNs_wt (left), ZFNs_mt (second-left), the CRISPR/Cas9 system containing the gRNA (second-right) and the CRISPR/Cas9 system without gRNA (Right). After 20 days, $eGFP^+$ cells were sorted and over 100 clones were grown in medium with neomycin for further analysis.

We designed a donor DNA fragment containing eGFP and Neomycin resistant gene expression cassettes flanked by 1kb homologous arms to the *WAS* locus (Figure 25A). The plasmids expressing the different endonucleases were nucleofected together with the donor DNA into K562 cells and the eGFP expression was analyzed at day 3 and 20 (Figure 25B). We achieved 30 to 45% $eGFP^+$ K562 cells 72 hours upon nucleofection (Figure 25B, top plots). Upon culture for 20 days between 0.3% and 6% of the K562 cells were stably expressing eGFP, in the case where nucleases were co-transfected (Figure 25B, bottom plots). These cells stably expressing eGFP, have integrated the donor DNA into the cells' genomic DNA. To investigate whether the integration has occurred in the *WAS* locus, we sorted the $eGFP^+$ cells and generated clones in the presence of neomycin in order to select those clones that have integrated the complete donor DNA.



	Total Clones	Out	In	% (In)
ZFN_wt	31	17	14	45
ZFN_mut	41	5	36	88
CRISPR/Cas9	35	6	29	83

Figure 26. WAS locus and donor modifications. A) The top diagram shows a scheme of the first 5 exons of the WAS locus (E1-E5) and the target site for ZFNs and CRISPR (top). The middle diagram shows the donor DNA used to edit the WAS locus containing the eGFP (Green) and the NeoR (Red) expression cassettes and the homology arms in 5' and 3' (in blue). The lower diagram shows the expected outcome of the edited WAS locus after homologous recombination with the donor DNA. The different primers are shown as black arrows and expected size of the different PCR products are shown with arrows of different colors. **B)** Representative analysis of the expected bands that should appear in clones in which the donor DNA has landed in the *WAS* locus (In Target: heterozygous WAS -/+ and homozygous WAS -/-) or outside the *WAS* locus (Out Target: homozygous WAS +/+). **C)** Table showing the specificity of donor integration with the different endonucleases. The Table shows the total number of clones analyzed, the number of clones containing donor integration outside WAS locus (Out) and the number of clones harboring donor integration at the *WAS* locus (In), as well as the percentage of clones harboring donor insertion at the correct site (% (In)).

To differentiate between donor integrations in the *WAS* locus (In-target) and integrations outside the *WAS* locus (Out target) a PCR was performed with primers described in figure 26A. The different clones, analyzed by PCR as depicted in Figures 26A and 26B, showed that ZFNs_mt and CRISPR achieved 88 and 83% of the insertions in the targeted locus, respectively (Figures 26B and 26C). In contrast, cells transfected with the donor DNA and the ZFNs_wt had only 45% of the insertions that landed inside the *WAS* locus, meaning that the majority of the inserts were outside of the target site (Figure 26C).

The above data are in line with the cutting specificity data of the different endonucleases obtained previously (Figure 22). To further confirm the integration of the donor DNA in the *WAS* locus, we analyzed WASP expression levels in homozygous and heterozygous clones by FACS analysis. Figure 27 shows the absence or reduction of WASP expression in homozygous and heterozygous clones, respectively.

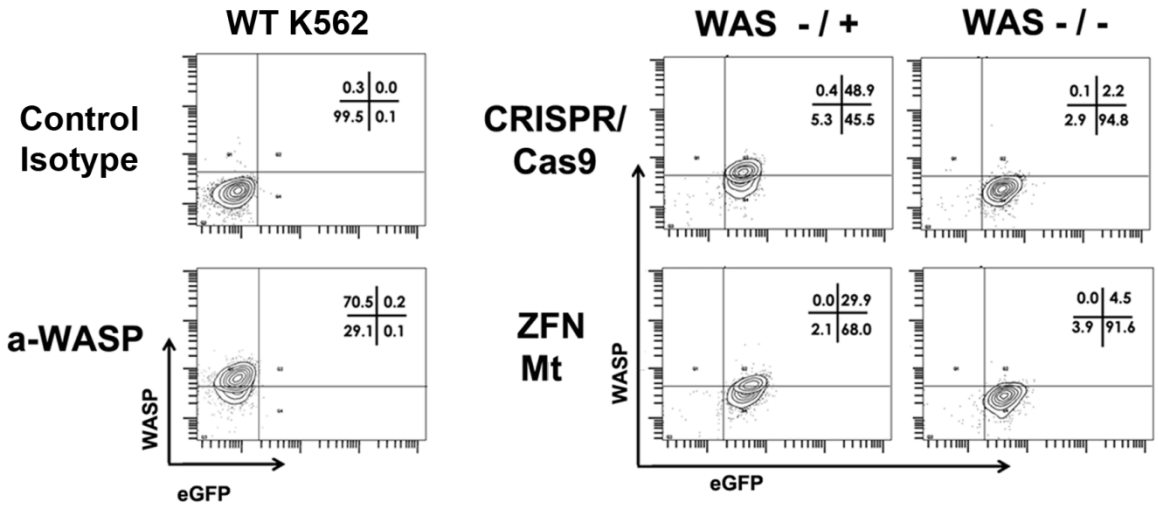


Figure 27. WAS expression after HR editing. WASP expression on K562 clones edited using CRISPR and ZFNs. **Left**: WT K562 cells stained with rabbit anti-WASP (abcam) antibody (bottom) or Isotype control (top). **Right**: Heterozygous (left; *WAS* -/+)) and homozygous (right: *WAS* -/-) clones for *WAS* gene disruption using the CRISPR (top) and ZFNs_mt (bottom). All clones express eGFP due to the integration of the Donor DNA. Heterozygous clones (*WAS* -/+) express around 50% of the WASP levels compared to WT K562 (49% and 30% compared to 70.5%) and homozygous clones (*WAS* -/-) showed no expression of WASP. Staining was as follow: 10^5 cells were washed with PBS, fixed with PFA (2%) and permeabilized with 0.2% saponine in PBS with BSA3%. Cells were then incubated with FcR, washed and incubated with rabbit anti-WASP antibody (1/100) or rabbit control (1/100) both from abcam. Secondary anti-rabbit alexa fluor 647 (Abcam) were added at a dilution of 1/1000. After washing with PBS, cells were analyzed for WASP expression using a FACSCanto II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with the FACS Diva analysis software (BD Biosciences).

5. Nucleofection delivery of ZFNs and CRISPR/Cas9 system into CD34+ cells.

Once we established the best system in the K562 cell line, we compared the different endonucleases into our target cells, the $CD34^+$ cells. We nucleofected $CD34^+$ cells with the two plasmids of ZFNs_mt and with the all–in-one plasmid containing the Cas9 and the gRNA (SC9Lg9W).

We also put a control eGFP plasmid in parallel to verify that the nucleofection was performed efficiently and obtained 40% GFP^+ $CD34^+$ cells upon nucleofection (Figure 28A). 48h post-nucleofection we harvested the cells and examined the cutting efficiency. For both system, ZFNs and CRISPR/Cas9, we were not able to reveal cutting at the *WAS* locus (Figure 28B). The absence of cutting efficiency could be due to the resistance of the HSCs to be nucleofected, especially since the endonuclease plasmids were quite big in size.

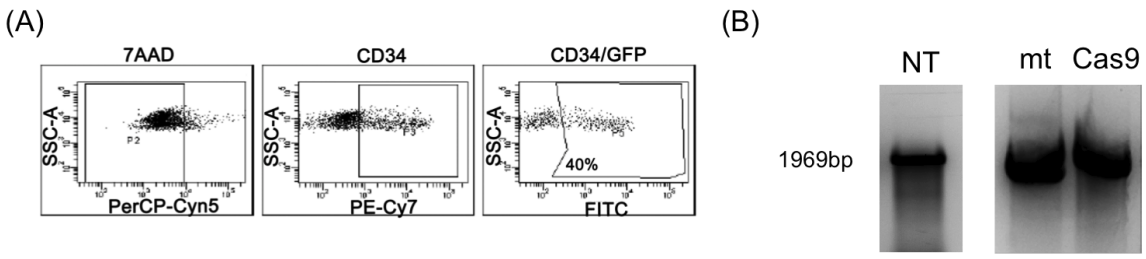


Figure 28. CD34$^+$ cells nucleofected with ZFNs and CRISPR/Cas9 system. **A)** Representative plot of GFP expressing cells 48h post nucleofection **B)** Representative electrophoresis gel, showing efficiency of cutting in CD34$^+$ cells after the surveyor assay (n>3).

6. IDLVs delivery of ZFNs and CRISPR/Cas9 system in HSCs.

Next, we used IDLV delivery system. As control we used IDLV-SE expressing eGFP from the same promoter as the ZFNs and CRISPR systems (the SFFV promoter). Using IDLV-SE, we observed up to 15% of transduced cells using a PNC of 500 (Figure 29A). We next transduced CD34$^+$ cells with IDLVs containing the two different vectors for ZFNs and SC9Lg9W at PNCs around 3000 each. However, we obtained a similar result as with nucleofection; we were not able to detect any cleavage for any of the different IDLVs (Figure 29B).

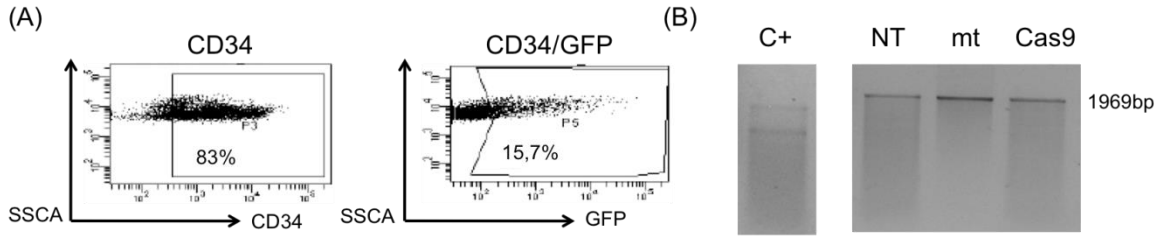


Figure 29. IDLV delivery system of ZFNs and CRISPR/Cas9 system to CD34$^+$. A) Representative plots of stimulated CD34$^+$ cells transduced with eGFP IDLVs. B) Representative electrophoresis gel, showing efficiency of cutting in CD34$^+$ cells after the surveyor assay (n>3). C+- positive control, NT- no transduce cells, mt- ZFN_mt and Cas9- SC9Lg9W.

We thought that the absence of WAS genome edition of CD34$^+$ cells with our IDLVs was probably due to the difficulty to obtain highly concentrated IDLVs and/or to their low expression levels. Improving the IDLVs could render better results, since IDLV-ZFNs have been shown to be able to edit CD34$^+$ cells by other groups.

We therefore focused on trying to improve the all-in-one CRISPR vector system by modification of the backbone and by pseudotyping with different envelopes.

7. Improvements in the IDLV delivery system

a. Improving the LV backbone: HS4 based insulators

IDLVs achieve usually low gene expression levels [149, 150, 242-245] due to their tendency to undergo epigenetic silencing [246, 247]. Different strategies have been pursued to improve titer and/or transgene expression of IDLVs.

The inclusion of insulators based on the chicken β-globin locus control regions (LCRs) hypersensitive 4 (HS4) has been somehow a successful approach. Even though their long size reduced the titers, they improve the transgene expression levels.

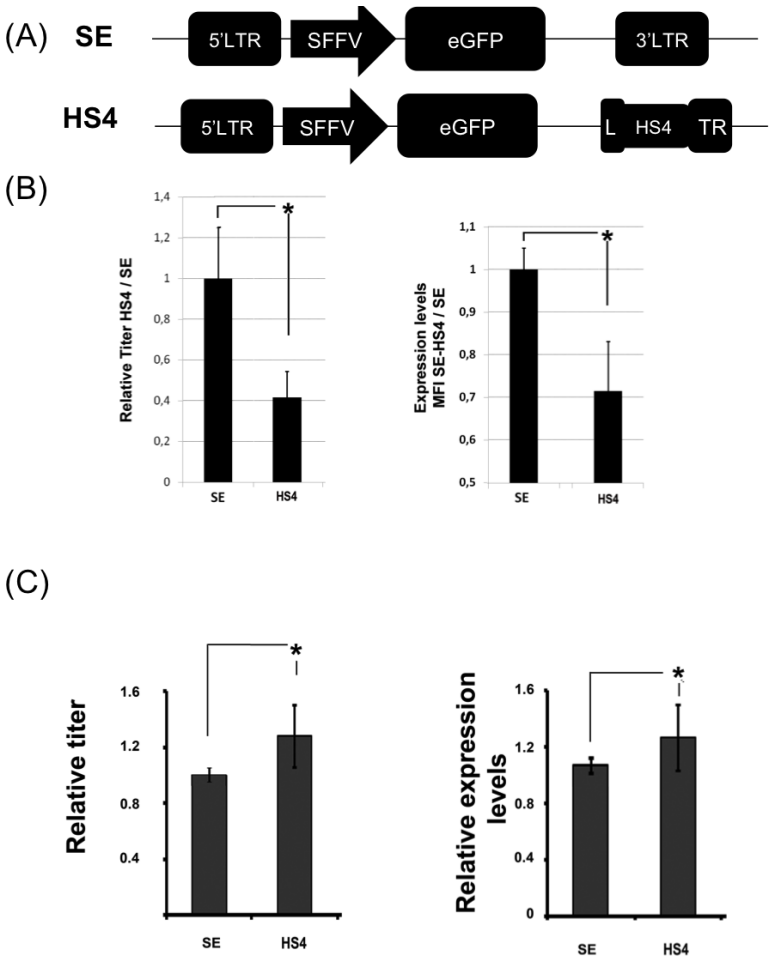


Figure 30. **HS4-based insulators**. **A)** Schematic diagram of the HS4 insulator and the LV constructed based on the SE LV. The HS4 elements were inserted into the U3 region of the 3' and the effect of the different HS4 elements on vector titer (Left) and transgene expression (right) compared to the SE LV **(B)** or SE IDLV **(C)**. Only populations having less than 15% eGFP+ cells were used for the analysis to avoid the effect of multiple LVs integrations. Values represent mean of at least three separate experiments and the error bar indicates the standard deviation of the mean. (* = $p<0.05$ Student's t-test; two tails, assuming equal variances).

In order to study whether these elements could enhance the titer and/or expression levels of IDLVs, we incorporated the HS4-core [152] into the LTR of the lentiviral vector backbone (Figure 30A). LVs and

IDLV particles of the SE and SEHS4-core were obtained and analyzed for titer and expression levels in K562 cells (Figure 30B and C, respectively).

Surprisingly, although we observed that the HS4-based integrating LV vectors suffer a significant decrease in titer and expression levels in K562 cells (Figure 30B), their SE-HS4c-IDLVs counterpart had increased titer and better expression levels compared to SE-IDLVs (Figure 30C). To gain a further insight into the nature of IDLVs which promote the high expression levels achieved using the insulated IDLVs, we quantified the different viral DNA genomes (total, 2LTR). The inclusion of the tested insulator enhances expression levels by favoring the formation of 1LTR rather than 2LTR molecules and this could be related to the negative effects that LTR modifications can have in retroviral integration. The next step in this direction would be to insert the HS4c element in the SCas9Lg9W backbone to see if the new IDLV (SCas9Lg9WHS4c) would be able to edit HSCs, since we could achieve higher titers and higher Cas9 expression levels (work in progress in the laboratory).

b. Adapting IDLVs pseudotyping to CD34+ cells and T cells

Another aspect that can be modified in order to improve the efficiency of IDLVs is the envelope used to generate the particles (pseudotyping). Different envelope glycoproteins can be used to generate LVs that target different cell types. We hypothesized, that the production of LV vectors with certain envelopes [133, 144] that improve the transduction of HSCs and T cells can also be applied to the non-integrative vectors.

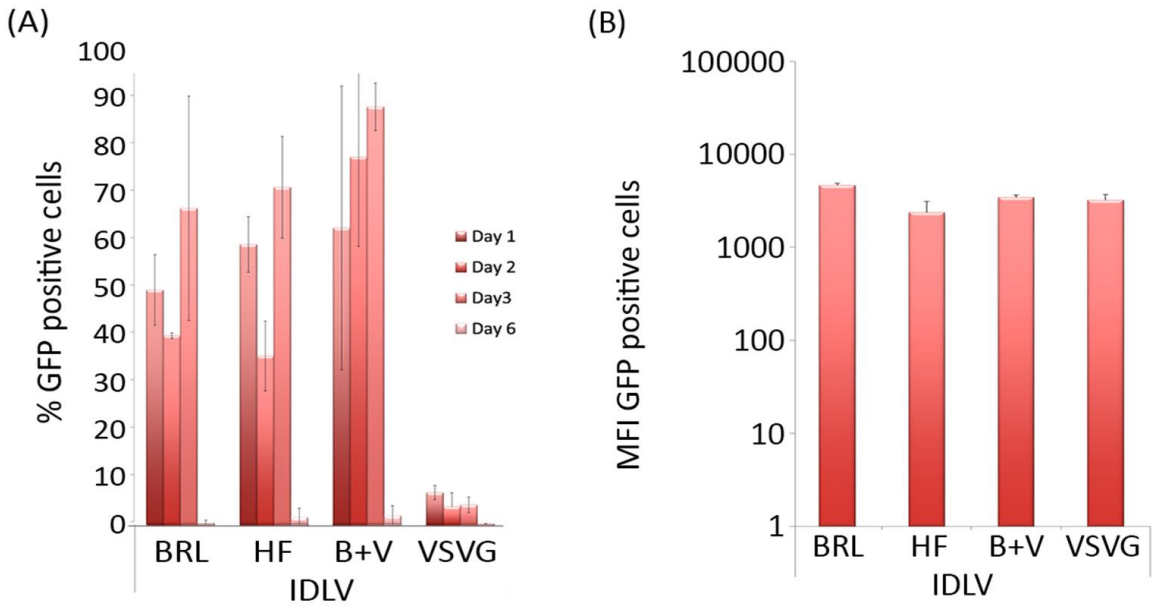


Figure 31. HF-LV/IDLV allows high levels of transduction in activated CD34+. Freshly isolated CD34+ from the same donor were stimulated with a cytokine cocktail (TPO, SCF, Flt-3) and subsequently transduced with BRL- and HF-IDLV GFP at MOI= 10 and BRL+VSVG- and VSVG-IDLV GFP at MOI 50. The percentage of GFP+ cells **(A)** and the MFI **(B)** of those cells was determined by FACS analysis (Mean ± SD, n=8) in logarithmic scale.

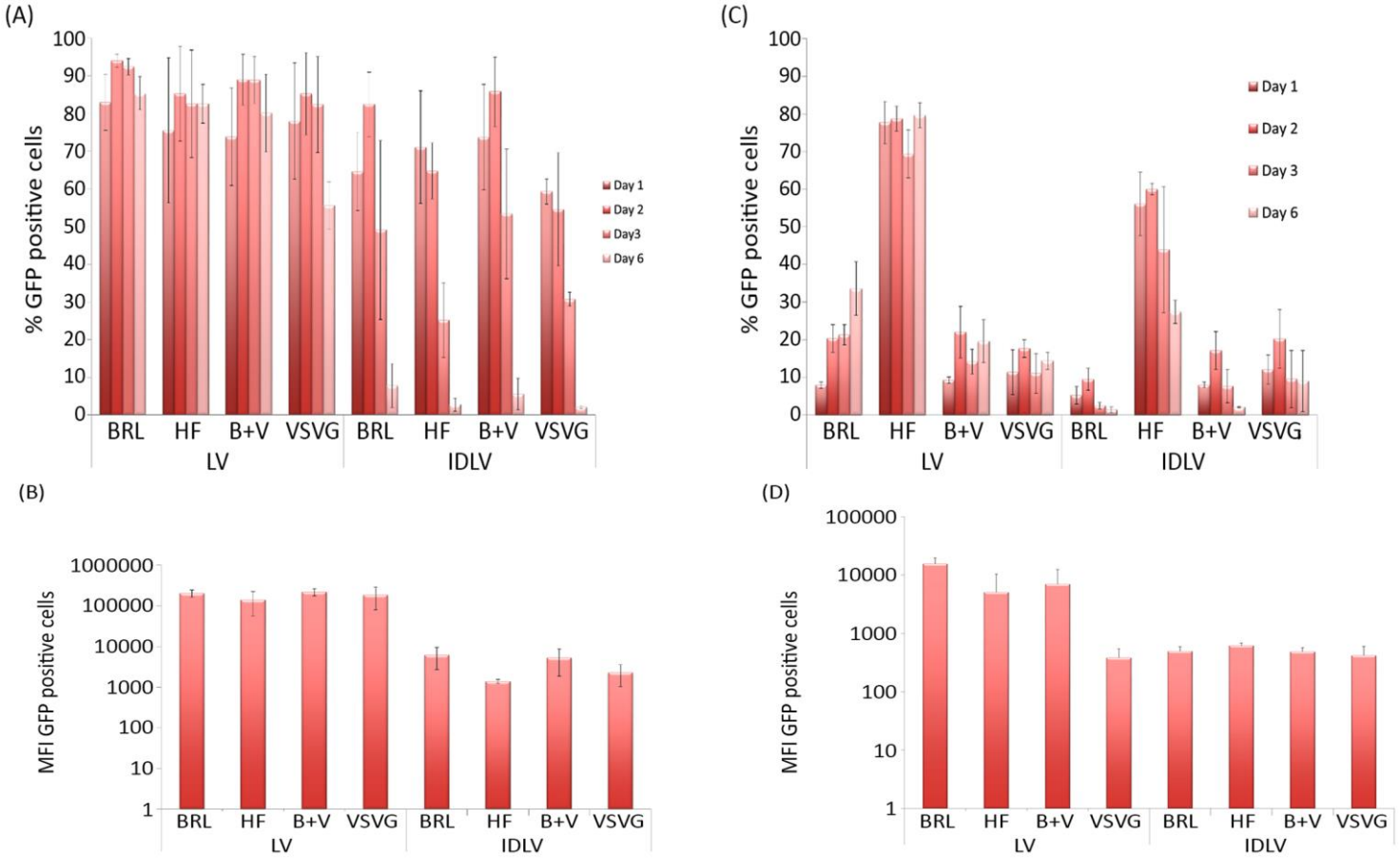


Figure 32. HF-LV/IDLV allows high levels of transduction in activated IL-7 T. Freshly isolated T cells from the same donor were stimulated through the TCR receptor or by IL-7 for 3 days and subsequently transduced with BRL- and HF- LV/IDLV GFP at MOI= 10 and BRL+VSVG- and VSVG- LV/IDLV GFP at MOI 50. The percentage of GFP^+ cells was determined in activated CD3/CD28 T cells **(A)**, activated IL7 T cells **(C)** and the MFI of those cells, CD3/CD28 T cells **(B)** and IL-7 T cells **(D)** by FACS analysis (Mean ± SD, n=8) in logarithmic scale.

In order to select the best envelope for HSCs transduction, we transduced $CD34^+$ cells, cultured under a cytokine cocktail (material and methods, with the SE vectors (expressing eGFP) pseudotyped with different envelopes; BRL, HF, VSVG and a mixture of BRL and VSVG using both integrative and non-integrative forms at the MOIs indicated in Figure 31.

Brieftly, BaEV wild type cytoplasmic tail was deleted for the R peptide sequence giving the BRL. The Edmoston's glycoprotein H and F were deleted for 24 and 30 aminoacids (aa) at their cytoplasmic tail and inserted under the control of the CMV promoter obtaining the plasmids encoding for H and F. As expected for what has been described previously using integrative LVs at these MOIs we observed low transduction levels of IDLV-VSVG (around 10%) while the IDLV-BRL and IDLV-HF transduced over 60% of $CD34^+$ cells. Remarkably, we observed that when we combined the BRL envelope with the VSVG the percentage of GFP^+ cells reached similar levels to those of BRL and HF alone.

Another interesting cell type for WAS gene therapy is the T cells. In order to select the best envelope for T cells, we transduced T cells activated with CD3/CD28 or with IL7 with the SE vectors ($eGFP^+$) incorporating different envelopes; BRL, HF, VSVG and a mixture of BRL and VSVG in both integrative and non-integrative form and at the MOIs indicated in figure 32. We observed that T cells activated by the TCR receptor pathway (CD3/CD28) were transduced at similar levels with LV or IDLVs pseudotyped with the different envelopes (Figure 32A). However, when the T cells are activated with IL7, a survival cytokine, the best performing envelope for both LV and IDLV transduction was the HF pseudotype (Figure 32) resulting in a 3- and 6-fold higher transduction, respectively, in comparison with the rest of the envelopes.

Taking all this data into account we used the best envelopes for each cell type when producing the IDLVs with the CRISPR/Cas9 system. However, when we tested the SC9Lg9W vector in either T cell or HSCs, we were not able to see any cleavage of the *WAS* locus (Figure 33), suggesting that there must be a reduction of IDLV transduction efficacy when the IDLV encodes for Cas9. However, since the number of particles produced by IDLV-Cas9 is very similar to the IDLV-SE (Table J), we hypothesize that the main problem of IDLVs-Cas9 could be the low Cas9 expression levels in the cell.

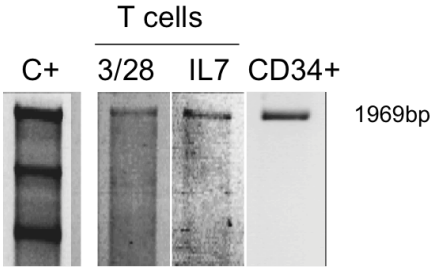


Figure 33. Pseudotyped-IDLV delivery of CRISPR/Cas9 system to $CD34^+$ and stimulated T cells. Representative electrophoresis gel, showing efficiency of cutting in $CD34^+$ cells after the surveyor assay.

8. A new CRISPR/Cas delivery tool: the Nanoblades

a. Development and characterization of HIV and MLV derived nanoblades

As an alternative to IDLVs, we next used VLPs that are called here nanoblades, which are designed to obtain a transient endonuclease delivery. They incorporate de Cas9 as a protein fused to the lentiviral or gammaretroviral structural protein gag and they form a VLP that incorporates the gRNA(s) (Figure 34) [165].

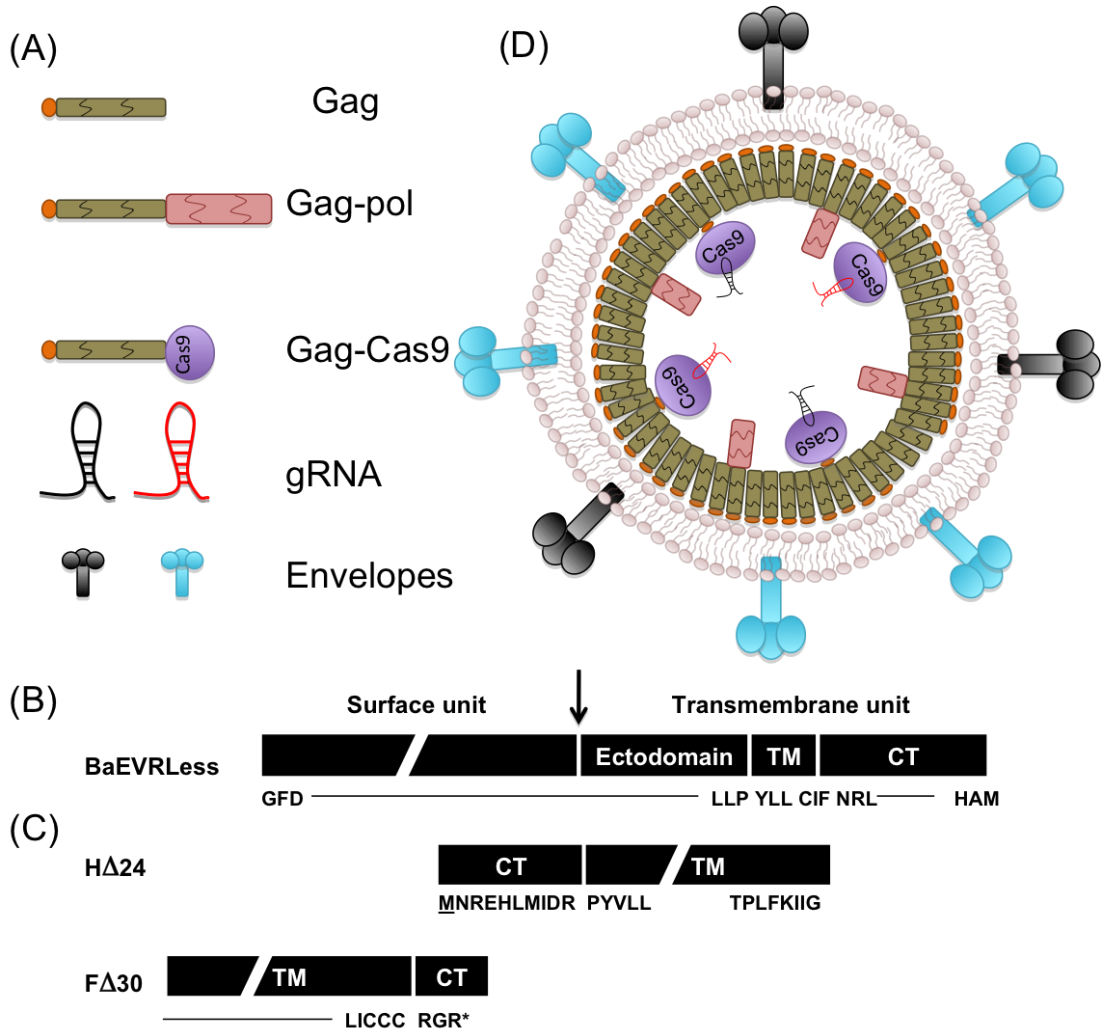
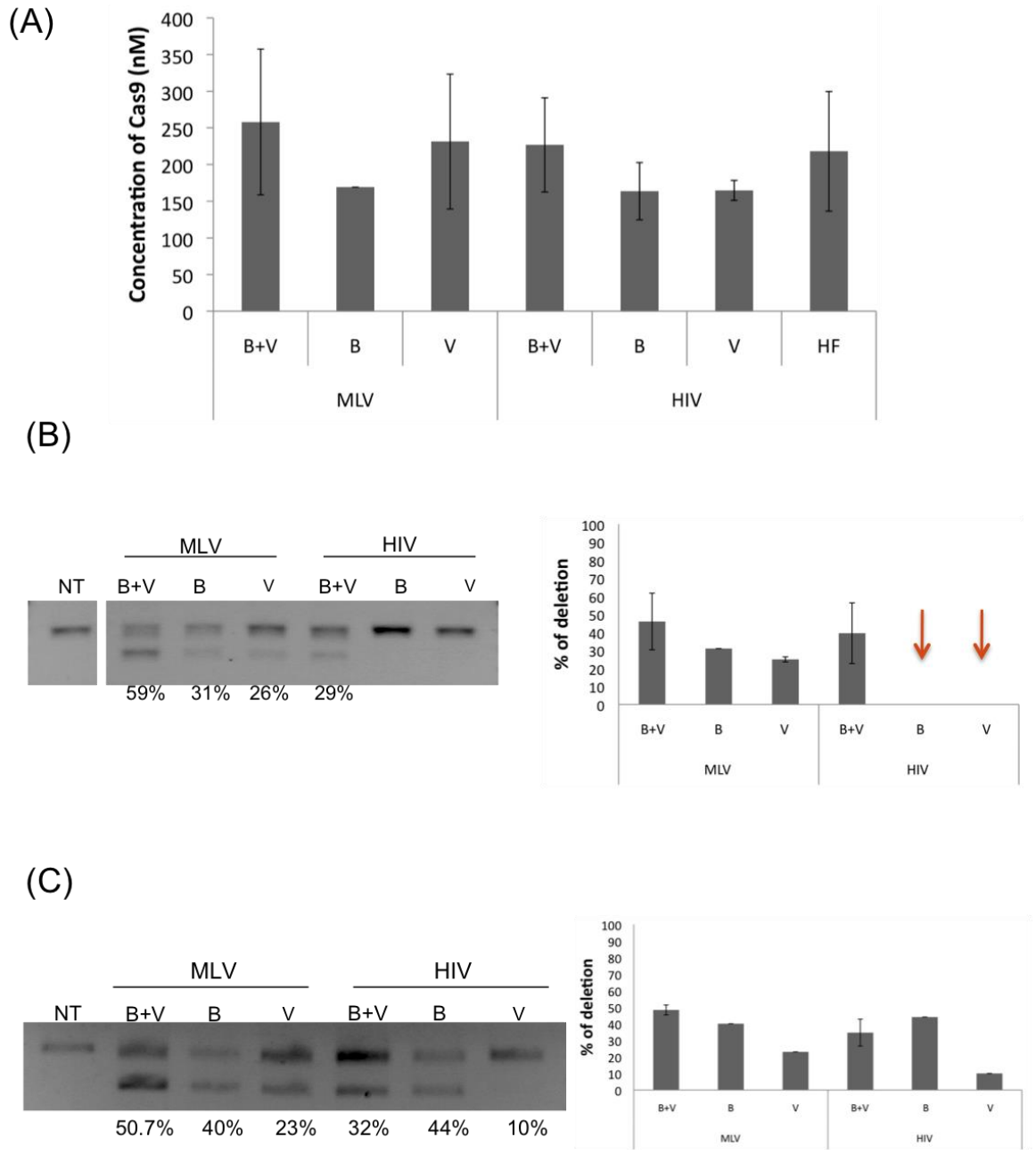


Figure 34. Schematic representation of the different components of the VLPs, and the envelope glycoprotein. **A)** Schematic of the different parts that compose the VLP. The Gag, the gag-pol, the gag-Cas9, the different gRNA and the envelopes. **B)** Schematic representation of the mutant BaEV (BaEVRless-BRL). The 17 amino acid long cytoplasmic domain was exchanged for the one of the MLV-A glycoprotein resulting in the chimeric BaEVTR-gps, and the R-peptide of the cytoplasmic tail of BaEVwt was deleted resulting in the BRL mutant gp. **C)** Schematic presentation of mutant F and H gps from the Edmonston MV strain. HD24 is a H mutant with deletion of the cytoplasmic tail. FD30 contains only 3 residual amino acids of the F wt cytoplasmic tail. CT: cytoplasmic tail, TM: transmembrane. **D)** Schematic of the assembly of the nanoblade particle.

The nanoblades are patented and were kindly donated by the group of Dr. Théophile Ohlmann (Eukaryotic and viral translation group) at the Centre international de recherché en infectiologie (CIRI, Lyon, France). The totality of the nanoblade experiments was performed during 2 consecutive short stays in the group of E.Verhoeyen, CIRI, Lyon.



Fiure 35. **Nanoblades efficiency in cell lines. A)** Graph showing the amount of Cas9 present in the VLPs by ELISA assay, (B-BRL and V- VSVG envelops) **B)** Efficiency of deletion of WAS gene in 293 cells and **C)** efficiency of deletion in WAS gene in K562 cells.

The nanoblades incorporated the Cas9, different to our OptCas9, in different backbones, MLV and HIV. They also incorporated two gRNAs targeting the exon 1: The gRNA 301 is located before the ATG of the exon 1 and the gRNA 305 is located at the 5' extreme of the exon 1 and finally they were pseudotyped with the different envelopes. The original nanoblades included the VSVG envelope [164] but we hypothesized that the inclusion of the Baboon envelope (BRL) would increase their specificity to transduce primary cells. We first transduced 293T cells with nanoblades containing the VSVG envelope, the BRL envelope and a combination of both envelopes: BRL and VSVG. We quantified the amount of Cas9 protein incorporated into the nanoblades by ELISA (Figure 35A) and observed that the VSVG envelope somehow increased the packaging of the Cas9gag fusion protein into the nanoblades, as we observed a higher amount of Cas9 protein, both in the MLV and HIV backbone when they carried both envelopes. Then, we tested the cleavage efficiency of the

different nanoblades into 293T cells (Figure 35B) and K562 cells (Figure 35C) by PCR of the *WAS* gene with the WASP2 primers (Table D) observing two bands: an uncleaved band of 811bp and another 647bp band that reflects the expected deletion of exon 1 if Cas9 cut in both gRNA target sites. We obtained around 50 % of deletion when both of the envelopes were present and around 20% when there was only one envelope. These data are in line with the amount of Cas9 observed in the ELISA (Figure 35A).

b. Nanoblades for gene editing at the WAS locus in primary human T cells

From the above data, we concluded that the co-display of BRL and VSVG on the nanoblades allowed the best delivery of Cas9 protein and the gRNAs as RNA into the cells. Therefore, we concluded that the nanoblades based on HIV and MLV pseudotyped with both BRL and VSVG envelopes would be the best choice to transduce primary cells. We pre-activated T cells either by the T cell receptor using anti-CD3 and anti-CD28 microbeads or by IL7.

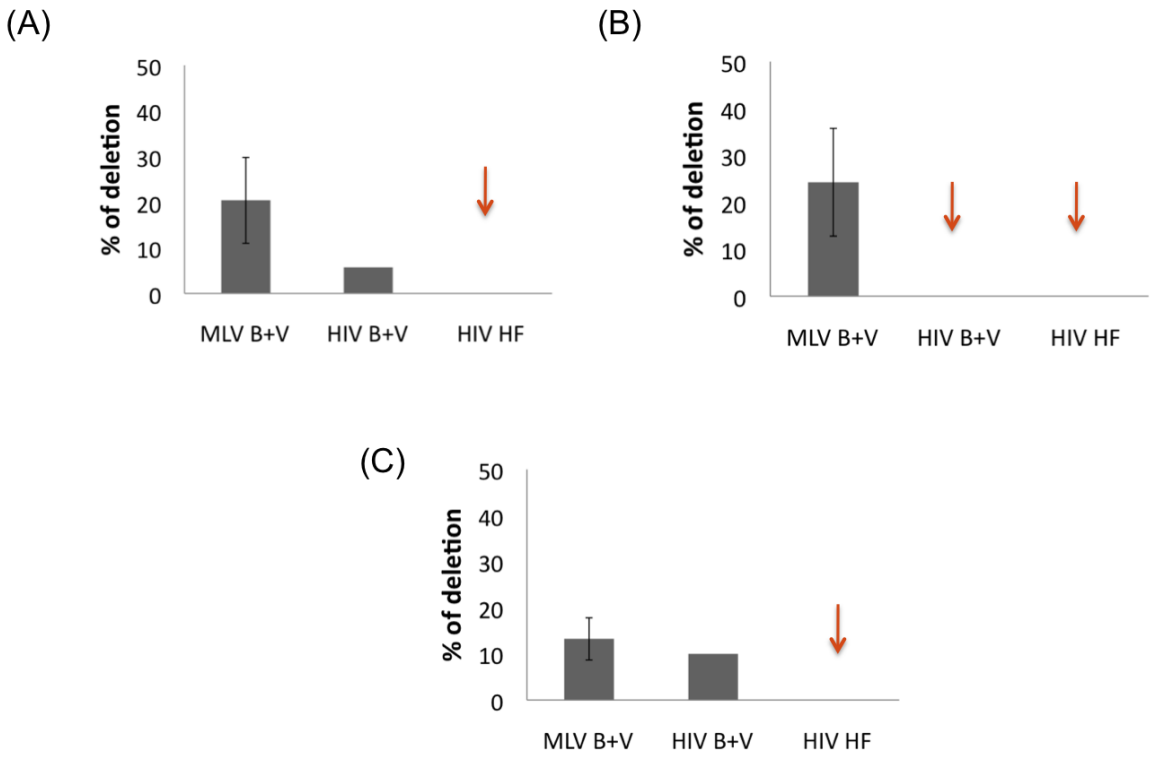


Figure 36. Nanoblades cutting efficiency in primary T cells. Graphs showing the percentage of dilution in *WAS* gene of **(A)** activated IL7 T cells, **(B)** Activated CD3/28 T cells and, **(C)** activated CD3/28 CD62L T cells measure by densitometry of the bands of electrophoresis gels after PCR of the WAS gene (n>2). Red arrows show no deletion detected when using 250, 230 220nM of Cas9 measured by Elisa for MLV B+V, HIV B+V and HIV HF, respectively.

After 3 days of activation we incubated them with the nanoblades and studied the cutting efficiency. We observed on average 20% editing with the MLV backbone while the percentage of the HIV backbone was consistently a bit lower, around 13% (Figure 36A) for CD3/CD28 activated T cells. However, this efficiency was lower in IL7 activated T cells, only reaching a 10% with the MLV backbone (Figure 36B).

We also isolated T cells expressing L-Selectin (CD62L) at their membrane, as it is known to be a subset of memory T cells strongly enriched in stem cell memory T cells (T_{SCM}) [248]. The gene therapy modification of these cells would lead to restoration of the *WAS* locus and expression of WASP resulting in functional T_{SCM} in WAS patients that can persist long term. After CD62L T cell isolation we activated them by the TCR receptor

and incubated them with the same nanoblades, observing similar gene editing efficiency at the *WAS* locus (Figure 36C) as compared to activated T cells (Figure 36A and B).

c. Nanoblades for gene editing at the WAS locus in primary human CD34+ cells

Since we obtained 50% of cutting with the nanoblade CRISPR/Cas9 system in K652 cell line, we decided to transduce primary human $CD34^+$ cells. For $CD34^+$ cells we attempted 3 different pseudotypes: HF, BRL and BRL/VSVG-LVs. All of these 3 envelope combinations incorporated in IDLV efficiently transduced $CD34^+$ cells (Figure 31) and T cells (Figure 32) confirming efficient entry of these IDLV pseudotypes into these cells types. However, because of the high efficiency obtained in T cells, we tested the MLV-based nanoblades pseudotyped with BRL and VSVG, while we used HIV-based nanoblades pseudotyped with BRL and VSVG or H /F in $CD34^+$ cells.

In $CD34^+$ cells the efficiency of cutting with the MLV derived nanoblades was better than in T cells, reaching up to a 30% (Figure 37). Although we obtained some *WAS* locus cutting with the HIV-based nanoblades the MLV BRL/VSVG nanoblades performed better (Figure 37).

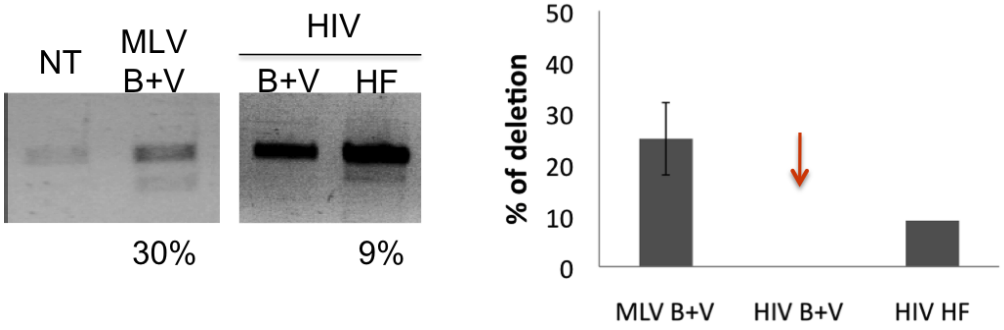


Figure 37. Nanoblades cutting efficiency in primary $CD34^+$ cells. Representative agarose gels after electrophoresis of *WAS* gene PCR of activated $CD34^+$ cells. The graph shows the mean of deletion percentage of each cell type (n>2). Red arrows show no deletion detected (n>3).

d. Nanoblades for gene editing at the WAS locus in CD34+ cell derived progenitor T cells

For the differentiation of $CD34^+$ cells into T cells (Figure 38A) we activated $CD34^+$ cells with a cytokine cocktail in a plate coated with DL4 and differentiated them for 14 days (Figure 38B). We observed how the cells changed phenotype from and early progenitor, $CD34^+/CD7^-$, to a progenitor T cell $CD34^-/CD7^+$ during the 14 days of differentiation (Figure 38C).

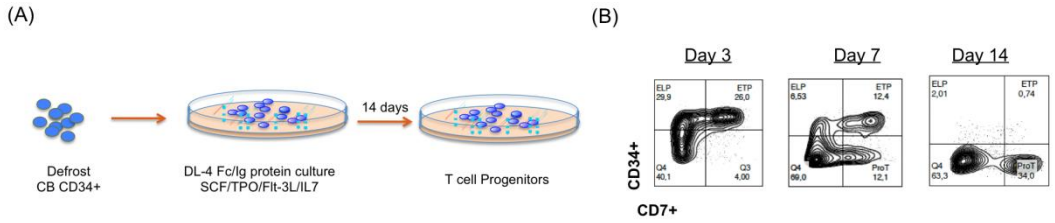


Figure 38 Pro-T cells differentiation from $CD34^+$. A) Scheme of the protocol for the differentiation of $CD34^+$ into pro-T cells. Cells were thawed and plated in DL4 coated plate with a cytokine cocktail and they were left to differentiate up to 14 days. **B)** Representative plots of the differentiation at different time points.

During this T cell differentiation process on DL4 coated plates, we transduced the cells at different days after the cytokine stimulation (Figure 39A) and observed by PCR the nanoblade deletion efficiency (Figure 39B) seeing a 10% with the MLV nanoblades at day 5 post differentiation. At the same time we transduced the progenitor T cells with IDLVs expressing eGFP at different time points of differentiation to determine the most suitable for transduction with an IDLV incorporating the donor template (Figure 39C) as well as to choose which envelope would be the one to give the higher expression. We saw that we achieved a 30% of eGFP positive cells at the different types of pro-T cells when transduced with the BRL envelope at day 4, not seeing any transduction with the rest of the envelopes and in the different days of transduction.

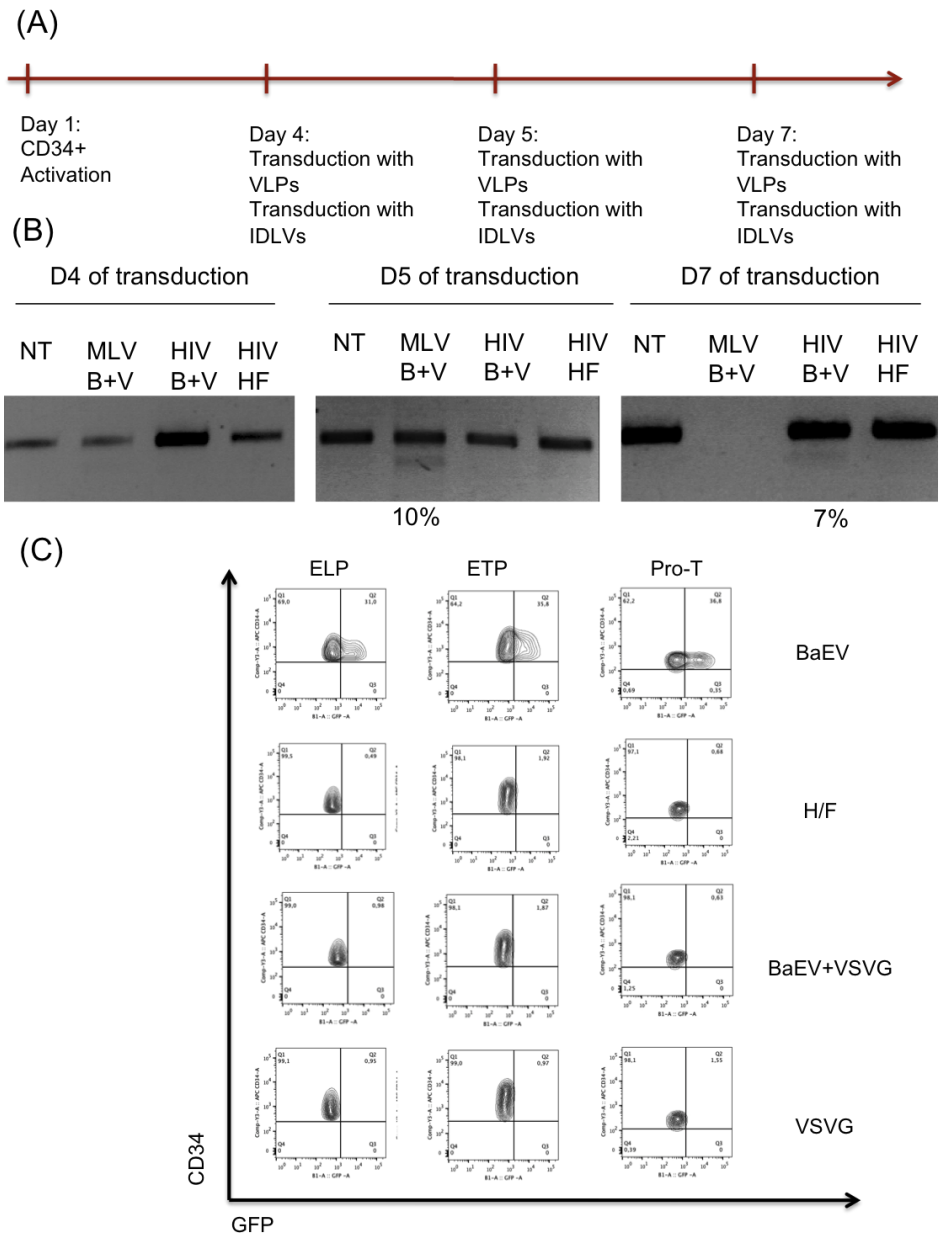


Figure 39 Optimization of pro-T cells transduction with nanoblades. **A)** Timeline of transduction protocol, **B)** Electrophoresis of the PCR amplification of the *WAS* gene with percentage of deletion at different days (day 4, day 5 and day 7), **C)** Representative plots of eGFP percentage of different pro-T cells types at different time points (day 4,day 5 and day 7). ELP- early lymphoid progenitors, ETP- early thymic progenitors.

e. *Off target effect of nanoblades*

To analyze the potential off-target effect of the nanoblades with the new gRNA we used the program CRISPRseek (http://www.bioconductor.org) [237] and selected all the possible off-target sequences for gRNA 301 and the 4 highest ones scoring for gRNA 305 (Table G and H and figure 40). We checked InDels generation in the genomic DNA of the different cells types (K562, $CD34^+$, stimulated CD3/28 or IL7 T cells and pro-T cells), in which we have seen deletion by amplification with the different primers against the different off-target sequences followed by surveyor assay and electrophoresis gel to see the cleavage. Nevertheless, we were not able to detect any off-target activity using this technology, most likely because the sensitivity of detection of the technique has to be above 2%. The use of whole genome sequencing, especially in $CD34^+$ cells might be more adapted for estimating off target activity of the WAS targeted nanoblades.

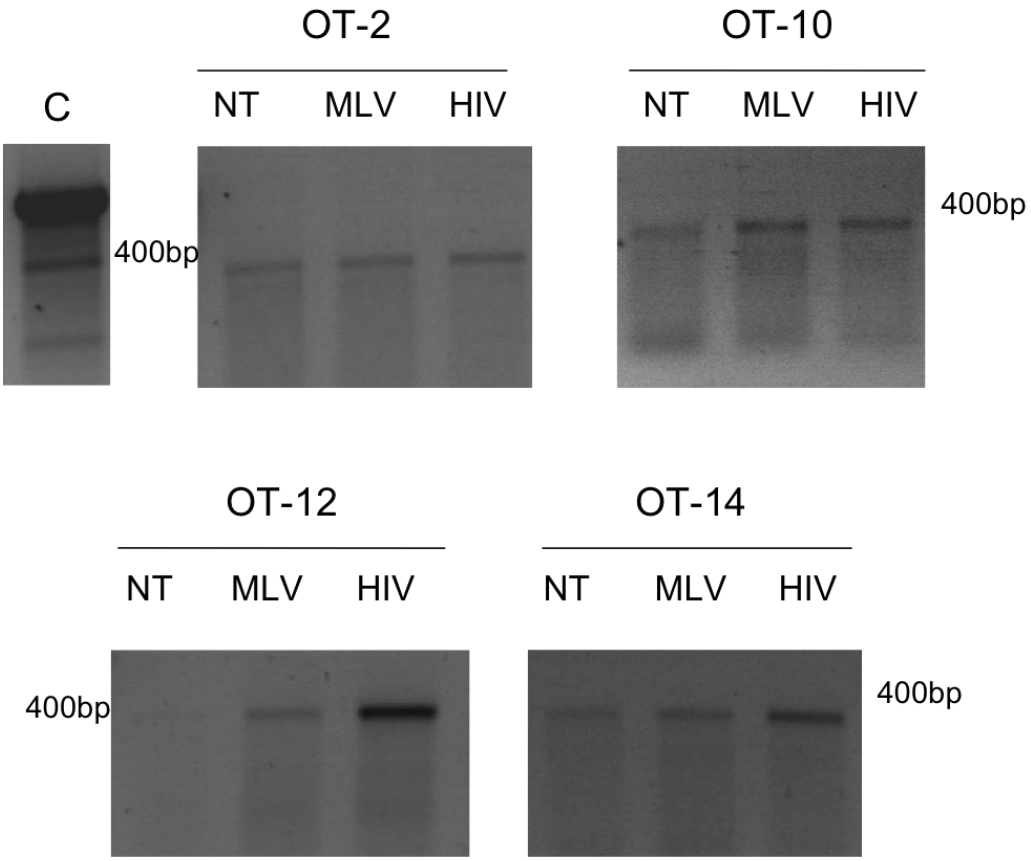


Figure 40. Potential off-target detection of CRISPR/Cas 9 nanoblades. Representative electrophoresis gel of the different off-targets tested in K562 cells. C+ is the positive control provided by the surveyor kit.

Discussion

"Science is a way of life. Science is a perspective. Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable - a transformation, for those lucky enough to experience it, that is empowering and emotional."

Brian Green

Discussion

Only recently, gene editing has emerged as a new gene therapy (GT) strategy that can use HR as well as NHEJ to treat different disorders. Nevertheless, although gene editing is very promising, it is not the Holy Grail yet, since it still needs several improvements before translation to the clinic. The fundamental parameters to improve are specificity, efficiency and fidelity of the gene editing tools. However, to be able to use them in the clinic not only all of them have to converge, but also improvements in the design of the specific nucleases, the donor and delivery efficiency of the tools need to be optimized.

Efficiency is bounded to the delivery of the endonucleases and/or donor DNA into the target cells, which most of the time depends on the cell type. The *ex vivo* transfection used with physical, non-viral methods, such as electroporation of DNA or mRNA into HSCs, has provided high transfection efficiency, however it is associated with cellular toxicity. In the case of viral methods, retroviral and lentiviral vectors, have also been used for *ex vivo* delivery into HSCs. Even thought they can be used in different tissues due to their broad tropism, both *ex vivo* and *in vivo* can induce long-term transgene expression, an effect not yet totally studied for nucleases. This is why transient expression of the nucleases is more desirable [249].

In this Thesis, we have tested different delivery methods: nucleofection, IDLVs and nanoblades in different cell types: K562 and primary cells; $CD34^+$ cells and T cells. The efficiency to deliver ZFNs has been enough to produce high percentage of cleavage in the K562 cell line, both by nucleofection and IDLVs; however it did not lead to any detectable cleavage in $CD34^+$ cells. We were able to observe a percentage of cutting with the dual version of the Cas9 by nucleofection similar to those achieved with ZFNs, however, the efficiency of the IDLVs was lower in comparison. We think that this might be due to quantity of the mRNA produced, as we observed that there was 3-5 times less CRISPR/Cas9 mRNA than ZFNs mRNA. We therefore designed an "all–in-one" vector, which included the Cas9 nuclease and the gRNA. This seemed to slightly improve the percentage of cutting in K562 cells with both nucleofection and IDLVs. In an attempt to further optimize gene editing, we decided to test different positions for the gRNA inside the vector and included the WPRE sequence (Woodcuck hepatitis virus Posttranscriptional Regulatory Element), which enhances the transgene expression. Even though the differences were not significant, the inclusion of the WPRE slightly improved the cutting efficiency by IDLV in K562 cells. The same observation was made when we positioned the gRNA into the LTR sequence. Therefore, we decided that adding the WPRE and positioning the gRNA in the LTR was the best vector to test specificity versus the ZFNs vectors.

As expected, optimized IDLVs were highly efficient for gene editing in the K562 cell line. However, we did encounter problems transducing $CD34^+$ cells with the IDLVs incorporating the CRISPR/Cas9 system. We decided first to try different envelopes for pseudotyping the IDLVs and LVs, as it has been shown that the VSVG protein, the most commonly used glycoprotein in LVs pseudotyping, is not ideal for transduction of human $CD34^+$ cells[250] nor T cell [120], potential target cells for WAS gene therapy. We therefore compared IDLV pseudotypes with different envelopes that have been proved to enhance transduction efficiency of these cell types (VSV-G, BRL and MV H/F). For the $CD34^+$ cells we observed that BRL and H/F enveloped lentiviral vectors transduced 6 to 8-folds better these target cells than the VSVG pseudotyped LVs and ILDLVs. What surprised us was to observe that the IDLVs that incorporated the BRL and the VSVG envelopes together showed an even higher transduction than the other envelopes alone. We hypothesized that the combination of the VSVG and the BRL envelopes is somehow improving the vectors entry through BRL receptors. When analyzing the T cells stimulated with anti-CD3/anti-CD28, there was no significant difference between the different IDLV pseudotypes although they seemed always slightly better than VSVG-IDLVs. However, in IL7-stimulated T cells (a situation that can improve gene therapy strategies based on T cells genetic modification) we clearly observed that IDLVs displaying the measles virus envelope (HF) gave over 60% transduction, which is 3-fold higher as compared to VSVG IDLVs, while the baboon endogenous retrovirus (BRL) enveloped IDLVs conferred the highest transduction levels (80% GFP^+ cells).

We therefore decided to test the IDLV/CRISPR pseudotyped with the BRL envelope for transduction of anti-CD3/28 stimulated T cells and $CD34^+$ cells, and with the HF envelope for IL7-stimulated T cells. However, we did not observe any cleavage in any of these cells. We hypothesised that this might be due to the expression of the transgene GFP (MFI) being 100-fold lower in IDLV as compared to integrating LV. This probably means that Cas9 expression is not sufficient for Cas9 activity to reach a minimum level leading to cleavage in the cells.

This is why we decided to use a new system based on VLPs, the nanoblades [164, 251]. We have observed that we are able to achieve around 50% of gene deletion in 293T cells and K562 cells. This efficiency of gene editing was similar to what we achieved in the same cell lines with concentrated all-in-one IDLVs-CRISPR system. Remarkably, CRISPR nanoblades achieved 30% and 20% cleavage at the *WAS* locus in $CD34^+$ cells and primary T cells, respectively. The nanoblades were, in our hands, the only tool which allowed efficient gene editing in primary hematopoietic cells. It might be due to the delivery of the Cas9 directly as a protein and the gRNAs directly as RNA. This might indicate that there is enough Cas9 in the cell at early time points of transduction to produce the cutting when the gRNAs are present in the nucleus. This means that the successful cutting of the nanoblades at the WAS locus was probably due to the level of the two components, Cas9 and gRNAs, in the cells at the same time in sufficient amounts.

Gene editing for its use in gene therapy has been growing in the last couple of years. So far in the current year, there have been more than 900 publications on this topic. The first clinical trials using specific nucleases as a therapy were performed using ZFNs, probably due to the fact that they were the first to be developed and have a quite high efficiency in HSC. There are different clinical trials ongoing using ZFNs targeting CCR5, a co-receptor of HIV. The purpose is to disrupt the CCR5 gene to treat HIV-1 patients by abolishing HIV entry in T cells and myeloid cells [81, 252].

Gene editing is also thought to allow specific TCR expression. This is based on the disruption of the endogenous TCR before expression of the new TCR which carries a specific anti-tumor recognition [253], therefore a more efficient CRISPR/Cas9 tool such as the nanoblades could mean an improvement for efficient TCR T cell modification.

Moreover, we were able to efficiently achieve disruption of the WAS locus in $CD62L^+$ T cells which are enriched in Tscm. Therefore, gene correction in these cell populations or CAR introduction in this particular population might provide a long-term cure since Tscm cells persist during the life time of an individual [254]. In the same line, we showed that progenitor T cells derived from HSCs were efficiently disrupted in the *WAS* locus. If we can achieve additionally also correction of an affected gene (ex. gamma C in the case of X-SCID or WASP in the case of WAS disease), T cell recovery might be much faster that upon correction of HSCs. This is important since the transplantation of these progenitor T cells would accelerate the reconstitution of T cell immunity as compared to introduction of corrected HSCs in a patient.

The **specificity** of a nuclease depends on its ability to target a specific site without modifying any other sequences of the genome. These may vary depending on the cell type, the nuclease used and the target locus. Therefore, the development of designed nucleases is focused on maximizing specificity; such is the case of obligate heterodimeric variants in ZFNs [63].

We wanted to corroborate the results obtained by Miller et al. using the WAS-specific ZFNs comparing the homodimer ZFNs pair versus the heterodimer ZFNs pair using nucleofection or IDLVs [63]. We first nucleofected K562 cells and at the same percentage of cutting we quantified the specificity of each system by measuring γ-H2AX. The phosphorylation of this key protein is used to detect DSB as it is recruited at damaged sites by the DNA repair machinery and then phosphorylated. It has been used as a biomarker for DNA damage and therefore was used to test the efficiency of drugs and for detecting tumours [238]. We observed, when

measuring the ɣH2AX by flow cytometry, that the homodimer ZFNs pair presents a higher percentage of ɣH2AX than the heterodimer ZFNs, corroborating Miller et al. findings. We also found that the CRISPR/Cas9 system has similar levels of those of the heterodimer ZFNs. When we tested the IDLVs vector in K562 cells we observed exactly the same thing, the homodimer ZFNs pair introduced 10-fold more damage than those observed in the heterodimer ZFNs and in the CRISPR/Cas9 system. However, to achieve the same percentage of cutting 2-4 times more vector of CRISPR/Cas9 was needed.

We used nucleofection and IDLVs to transduce $CD34^+$ (HSCs) with our optimized CRISPR/Cas9 system and the heterodimer ZFNs pair. Although, we obtained efficient transduction with a GFP encoding plasmid or vector, we were not able to see any cutting efficiency when using the nuclease encoding plasmids or vectors. For nucleofection, it might be due to the use of plasmids, it has been demonstrated that the use of mRNA nucleofection gives high efficiency of cleavage in $CD34^+$ cells [84]. In the case of the IDLVs, it is probably due to the lower expression of the episomal forms [147]. It has been reported that IDLVs are organized into nucleosomal structures, enriched in histone modifications. This is characteristic for silenced chromatin resulting in transcriptional silencing of gene expression [255]. This is why the inclusion of insulators might help to improve the gene expression as they avoid silencing of the transgene [152]. We have confirmed this in K562 cells with lentiviral vectors and IDLVs, expressing the *GFP* gene. Insertion of insulators could be used to improve the expression of the Cas9, but it also could be used in the delivery of a donor DNA by IDLVs.

The **fidelity** of genetic restoration implies that the donor DNA integrates appropriately in the targeted locus and that no insertion of the donor DNA occurs outside it. Thus, HR mediated by nucleases depends not only on the efficiency of the nuclease but also on the correct insertion of the donor DNA in the target site. The fidelity could be compromised by the insertion of the donor DNA into a non-homologous site using DSB generated by the normal activity of the cell or by DSB generated by off-target activity of the endonucleases. In addition, it has been shown that populations exposed to nucleases not all of the targeted alleles are going to follow HR after the DSB, but they are going to be disrupted. This is due to the fact that during the cell cycle NHEJ pathway is activated and competes with the HR when DSB are produced [256]. To overcome the preference for the NHEJ pathway, the use of nickase nucleases, which only generate single strand breaks, has shown to trigger the HR pathway rather than the NHEJ [257, 258].

We have shown here that we were able to introduce into the *WAS* locus a donor template expressing GFP into K562. As expected, we observed higher levels of donor integration outside the *WAS* locus (55%) when the ZFNs_wt pair than the ZFNs_mt pair (20%). Interestingly, the CRISPR/Cas9 system showed similar behaviour compared to the ZFNs_mt, indicating that a good design of the gRNA can achieve similar fidelity than the heterodimeric ZFNs for HDR gene insertion. This 20% of out-target insertion could be caused by several reasons. One of them is an existent break that the cells overcome while divining and at the moment of the repair they come across the donor template and they use it. These insertions are random and do not have anything to do with the nuclease unspecific cleavage, as we have already seen that there are basal levels of H2AX.

Some pre-clinical trials have been launched using gene editing for monogenic diseases, such as X-SCID [84]. The first clinical trial using CRISPR started in November 2016 [259, 260]. It has been done in an aggressive lung cancer, where patient T cells are modified with CRISPR to disrupt a gene, PD-1, which will reactivate the immune response due to interruption of PD-1 binding to PDL-1, making these modified T cells able to target and kill the cancer cells [261].

There is still a lot of optimization needed to improve gene editing tools, delivery and specificity. Now that we have been able to achieve nearly a 30% of cleavage in primary T cells and $CD34^+$ cells we need to be able to

introduce the corrected *WAS* gene into these modified cells. Once this is done *in vitro*, the next step will be the engraftment of these cells in NOD/SCID gammac-/- mice to confirm correction in all the different lineages.

As we have seen, the inclusion of insulators increases the expression levels of IDLVs. In this sense, maybe their inclusion together with Cas9 and the gRNA would give enough expression of Cas9 to be able to produce a cleavage in primary cells. However, new techniques, such as electroporation of mRNA or Cas9/gRNA ribonucleoproteins, or even the nanoblades we have studied in this thesis, show higher efficiency for gene editing. Nevertheless, Cas9 IDLVs with insulators are promising for *in vivo* genome editing.

In summary, we have been able to create a new CRISPR/Cas9 system for the *WAS* locus with good efficiencies and specificities using plasmid nucleofection or all-in-one IDLVs. However, the heterodimer ZFNs pair appears to be the best alternative if IDLVs are planned to be use as a method of delivery. Nevertheless the new approach with nanoblades is a more efficient alternative to CRISPR/Cas9 IDLVs, especially in primary cells such as T cells and HSCs.

Conclusiones / Conclusions

"It is often said that science must avoid any conclusions which smack of the supernatural."

Michael Behe

Conclusiones

1. El sistema CRISPR/Cas9 y las ZFNs optimizadas son similares en cuanto a eficiencia y seguridad en la edición génica de WAS.

2. Los IDLVs de ZFNs son más robustos que los IDLVs de CRISPR para la edición genética.

3. La introducción del aislador HS4c en el *esqueleto* del vector lentiviral mejora la eficiencia de los IDLVs.

4. El pseudotipaje de los IDLVs con las envueltas HF aumenta la eficacia de transducción en las $CD34^+$ y en las células T activadas en comparación con la envuelta VSVG comúnmente utilizada.

5. La entrega de las endonucleasas utilizando IDLVs requiere más optimizaciones para editar HSCs y células T, probablemente debido a los bajos niveles de expresión.

6. Las nanoblades CRISPR/Cas9 pesudotipadas son capaces de introducir eficientemente Cas9 y los gRNA en $CD34^+$ y células T y son una herramienta interesante para terapias futuras del gen *WAS*.

Conclusions

1. The CRISPR/gRNA9 and the optimized ZFNs have similar efficiency and safety for WAS genome edition

2. IDLVs-ZFNs are more robust than IDLVs-CRISPR for genome editing.

3. The introduction of the HS4c insulator into the LV backbone improves the efficacy of IDLVs.

4. The pseudotyping of the IDLVs with the HF envelope increased its efficacy of transduction in $CD34^+$ and activated T cells in comparison to the commonly used VSVG envelope.

5. The delivery of endonucleases using IDLVs requires further optimization to edit HSCs and T cells, probably due to low expression levels.

6. Pseudotyped CRISPR/Cas9 nanoblades deliver efficiently Cas9 and gRNAs into $CD34^+$ and T cells and are therefore a interesting tool for future WAS gene therapy approaches.

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"The most important function of a bibliographic entry is to help the reader obtain a copy of the cited work."

Daniel J. Bernstein

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