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Gene Editing in Adult Hematopoietic Stem Cells

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Additional information is available at the end of the chapter

Abstract

Over the last years, an important development has allowed the scientific community to address a precise and accurate modification of the genome. The first probe of concept appeared with the design and use of engineered zinc-finger nucleases (ZFNs), which was expanded later on with the discovery and engineering of meganucleases and transcription activator-like effector nucleases (TALENs) and finally democratized and made easily available to the whole scientific community with the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease combination technology. The availability of these tools has allowed a precise gene editing, such as knockout of a specific gene or the correction of a defective gene by means of homologous recombination (HR), taking advantage of the endogenous cell repair machinery. This process was already known and used but was inefficient—efficiency that has been increased more than 100-fold with the addition of the mentioned specific nucleases to the process. Apart from the proper design of the nucleases to recognize and cut the selected site in the cell genome, two main goals need to be adequately addressed to optimize its function: the delivery of the tools into the desired cells and the selection of those where the gene editing process has occurred correctly. Both steps can be easily solved when the source of cells is extensive or can be expanded and manipulated *in vitro* extensively, such as immortalized cell lines or pluripotent stem cells (embryonic stem cells and induced pluripotent stem cells). However, both steps are critical in the case of primary cells, such as the hematopoietic stem cells (HSCs). HSCs are a rare cell population present in the bone marrow (BM) of higher mammals, and it is the responsible for the maintenance and

replenishment of all hematopoietic cells for the lifespan of the animals by means of two fundamental properties: self-renewal and multipotency. HSC population is then the ideal target for the correction of hematopoietic genetic diseases and also for the knockout of the responsible genes to *in vitro* and *in vivo* model those hematopoietic diseases. This rare population cannot be expanded and its *in vitro* manipulation and culture negatively affects their fundamental properties of self-renewal and multipotency. These factors challenge the application of gene editing to HSCs. Important efforts are now ongoing trying to optimize the protocols of gene delivery and selection for HSCs. This chapter will review and discuss how researchers are trying to solve them, all attempts that are ongoing and the potential application of the technology to the patients affected with hematopoietic genetic diseases.

1. Introduction

Adult hematopoietic stem cells (HSCs) are a rare population of cells that are present in the bone marrow (BM) and are the responsible for the generation of all mature blood cells, including erythrocytes, platelets, and immune cells (1). This population represents less than 0.1% of the total BM and is the only one capable of self-renewal, being responsible for the maintenance of the lifelong hematopoiesis. Since their definition at the beginning of the last century (2), an extensive knowledge of this population has been accumulated, being probably the best known adult stem cell. HSCs can be phenotypically and functionally characterized and can be purified and even manipulated *in vitro*. Once transplanted back into the animal or human body, HSCs are capable of regenerating the whole hematopoietic system of the organism (1,3). These capabilities established the possibility of BM transplantation as a therapeutic strategy for the treatment of hematopoietic diseases, either inherited or acquired. BM transplantation can be autologous, when the HSCs come from the same person, or allogeneic, when they come from a healthy donor. Allogeneic BM transplant is the only curative treatment for oncogenic diseases and for inherited (genetic) diseases, providing healthy HSCs. However, the immune reaction of the transplanted cells against the recipient [graft-versus-host disease (GVHD)] and the availability of compatible BM donors reduce dramatically its applicability and increase the severe adverse effects of the treatment (4). To definitively solve this problem, the genetic modification of the autologous HSCs to restore the genetic defect or to provide them with new capabilities, the so-called gene therapy, appeared as an additional therapeutic option avoiding GVHD and providing repaired/cured cells.

The idea of gene therapy appeared up in the middle of the 20th century, when the use of modified viruses as vectors to introduce the desired genetic material into the desired cells was already mentioned and demonstrated as a proof-of-concept (5). Since then, gene therapy has been playing in a roller coaster with tremendous improvements and successes but also with serious problems that have been dramatic in some instances (6). Finally, gene therapy is nowadays established as a real therapeutic option for a number of hematopoietic inherited

diseases, such as immunodeficiencies or hemoglobinopathies (7). The use of self-inactivating lentiviral vectors, the improvement of the *in vitro* culture conditions of the human HSCs, and the better knowledge of HSC engraftment in the recipient has contributed to this reality (8). Nevertheless, HSC gene therapy still has some drawbacks that needed to be addressed to make this strategy safer. The most important one is the potential insertional mutagenesis that could derive in an oncologic problem due to the inability to control the insertion site of the genetic material using the present available tools (8).

Precise gene editing using the gene repair cellular machinery appeared as the next step to follow. Widely used in the generation of animal models, mainly fish and mouse, its application as a therapeutic option in humans was far to be considered due to the low efficacy and the difficulty to select those cells where the desired gene edition had taken place. The discovery of enzymes and biological systems able to cut in one precise and specific sequence of the genome (nucleases and nuclease systems) and activate the endogenous DNA repair pathways has clearly approached gene editing to be considered as a potential therapeutic approach in the near future. First tested for their therapeutic potential for hematopoietic genetic diseases in induced pluripotent stem cells (hiPSCs) (9), the principal bottleneck in these experiments was the generation of functional HSCs from the edited hiPSCs. Thus, an increasing number of laboratories, including ours, are now adapting the gene editing protocols to adult HSCs. Important steps, such as the delivery of the gene editing tools, improving protocols to maintain HSCs *in vitro* to allow their proper edition, and improving systems to select properly edited cells, are important barriers that need to be overcome to definitely apply gene edition-based gene therapy to the clinics. Some of the steps that are now being taken are explained below.

2. Hematopoiesis and hematopoietic stem cells

The hematopoietic system is responsible for the production and maturation of the different components of the blood in a process called hematopoiesis. Blood is one of the most active regenerative tissues, with approximately 1 trillion mature cells arising daily in adult human BM (3). The hematopoiesis is a highly hierarchically organized structure in which all types of mature blood cells are generated from a small subpopulation of undifferentiated cells allocated in the BM. The hematopoietic system is divided in two main different lineages: lymphoid and myeloid. The lymphoid lineage gives rise to the T cells, B cells, and natural killer (NK) cells, whereas the myeloid lineage formed granulocytes, monocytes, erythrocytes, and megakaryocytes/platelets (1). Different organs and tissues are implicated in the production and homeostasis of the hematopoietic systems, such as blood, BM, liver, spleen, thymus, and lymph nodes.

HSCs are the most primitive cells in the hematopoietic system and support all the hematopoiesis. They are characterized by their capability to self-renew and to produce all types of mature blood functional cells. These cells persist throughout adult life, maintaining their hematopoiesis (1). Hematopoietic committed progenitor cells, the next step in the hematopoietic differentiation process, arise from the HSCs. These cells have a higher proliferative activity than HSCs, a limited self-renewal capacity and the ability to differentiate a limited

number of mature cell types. Finally, mature cells complete the pyramid structure of the hematopoiesis. These cells are characterized by a recognizable morphology, full functional capabilities, and a low or null capacity of proliferation and self-renewal (10).

The study of human HSCs began with the identification of colony-forming progenitors using *in vitro* CFU-C assays (11–14). Nowadays, human HSC capacity is usually tested in xenogeneic transplants, in which human HSCs are transplanted in immunodeficient mouse strains that have a permissive microenvironment for the human HSC engraftment (15). The first human-ized mouse model was the severe combined immune-deficient (SCID) mouse lacking B and T cells (16,17). Then, different mouse models were developed based on their ability to efficiently support high levels of all lineages of human engraftment (18–20). The CD34 marker was identified as a relevant indicator of human hematopoietic progenitors, which includes HSCs (21,22); nevertheless, due to the variability in their repopulation ability, several subpopulations of HSCs were identified in xenograft models, some subpopulation with a transient and limited ability to support the hematopoietic system, such as short-term HSCs (ST-HSCs), multipotent progenitors (MPP), committed progenitors, or long-term HSCs (LT-HSCs), which are able to maintain a durable hematopoiesis (23,24).

Only 1 in 10^6 in human BM is a transplantable HSC (25); thus, the scarcity of HSCs has led to the necessity to purify the HSCs from the heterogeneous bulk population to allow their handling and manipulation. The purification of HSCs requires the detection of several cell surface markers. There are several markers whose expression is gained or lost during the differentiation process. In mice, HSCs do not express lineage markers (myeloid or lymphoid), so they are called lineage negative cells. In addition, HSCs express high levels of the stem cell antigen 1 (Sca-1) and c-Kit (CD117) marker (**Figure 1**). These cells are called LSK cells (lineage negative, Sca-1 positive, c-Kit positive) (1,26,27). As in mice, the absence of all lineage markers (Lin⁻) determines human HSCs (28). As previously mentioned, CD34, expressed on 0.5% to 5% of all blood cells, was the first marker found that identifies human LT-HSCs and more differentiated progenitors (29). Later on, other markers were described in LT-HSCs such as the expression of the CD90 (Thy1) (30) or the lack of expression of CD45RA and CD38 (found to be expressed in more differentiated progenitors) (31–33). Moreover, integrin CD49f (involved in cell attachment to the extracellular matrix) was shown to be expressed in LT-HSCs (34). With respect to ST-HSCs or MPP, the loss of Thy1 and CD49f expression was proposed to be a key signature through a more differentiated state (3). Consecutive studies have classified the different populations depending on the expression of alternative markers, such as the expression of aldehyde dehydrogenase (ALDH), and other surface markers, such as CD117 or CD133 (35).

3. Gene correction of hematopoietic stem cells by addition strategies and their drawbacks

Gene therapy can be achieved by the delivery of genetic material into cells affected by a disease, and it can be accomplished by the addition, substitution, or alteration of the related genes (8). These modifications can be carried out by *in vivo* direct infusion of a vector having the ectopic
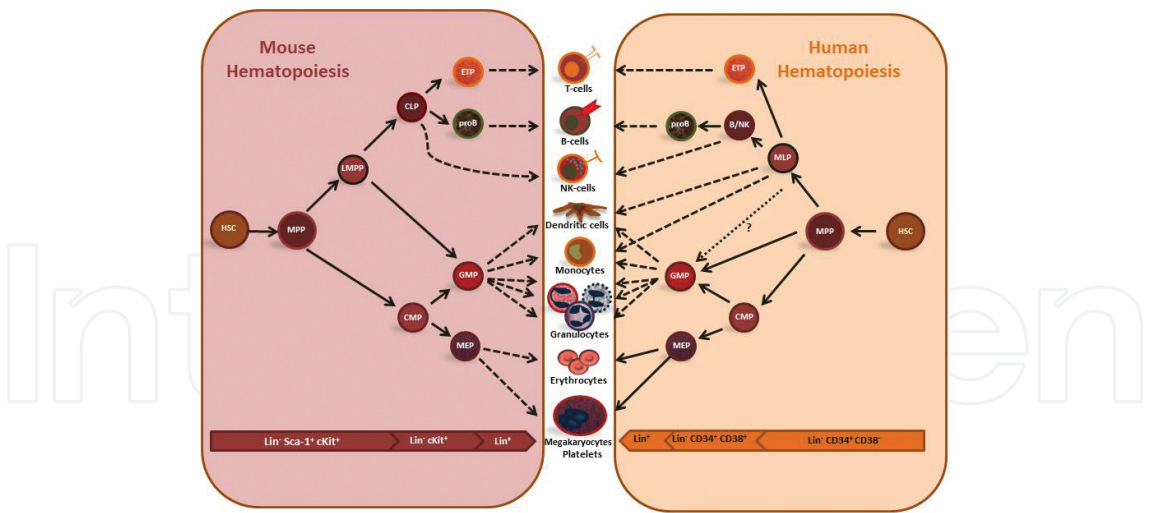
Figure 1. Human and mouse hematopoieisis. Similarities in terms of antigen differentiation markers.

therapeutic gene or by *ex vivo* manipulation of patient's cells and their reinfusion into the patient. Gene therapy has become a feasible and attractive alternative therapeutic strategy for several diseases, from inherited hematopoietic, immune, and nervous diseases, including primary immunodeficiencies, leukodystrophies, thalassemias, hemophilias, and retinal dystrophies to cancer and other malignancies (36). In the case of blood-related diseases, HSCs have long been the preferred target for *ex vivo* gene therapy due to the feasibility to isolate them *ex vivo*, genetically correct them and reinfuse them through intravenous infusion (37,38). The correction of HSCs is a promising therapeutic approach that can provide a steady and stable expression of the transgene, restoring the function of the malfunctioning gene in the patient. Stable expression of the transgene can be obtained by addition gene therapy using vector-mediated transgene insertion (**Table 1**).

Disease	Vector	Patients	Conditioning regime	Target cells	Transduced cells	Outcome	Adverse effects	References
ADA	γ-RV	Italy (18), USA (16)	Busulfan	Lymphoid cells and NK cells	HSCs	Transient or no clinical benefit. Three patients with clonal expansion with long-term efficacy followed by inactivation of the transgene	No	(41,151–153)
		UK (8)	Mephalan or busulfan					
	SIN-LV	UK (10), USA (10)	Busulfan			Unpublished, ongoing	No	—

Disease	Vector	Patients	Conditioning regime	Target cells	Transduced cells	Outcome	Adverse effects	References
SCID-X1	γ-RV	UK (11), France (11), USA (3)	None	Lymphoid cells and NK cells	HSCs	Significant T-reconstitution/T-ALL in 5 patients, 1 dead	Yes	(40,42,47,154–157)
	SIN γ-RV	UK, France, USA (9)	None			Reconstitution of functional T cells but at low number	No	(158–160)
	LV	USA	Busulfan			Ongoing		—
CGD	γ-RV	USA (10), and (3)	None or Busulfan	Neutrophils	HSCs	No benefits	Yes	(43,161–168)
		Germany (2)/ Switzerland (2)	Busulfan			Long-term correction 4/4		
		UK (4)	Melphalan			Transient correction		
		Korea (2)	Busulfan +fludarabine			Short-term correction		
	SIN γ-RV	Germany	Busulfan			Ongoing		—
	LV	UK, Switzerland, Germany, France, USA	Busulfan			Ongoing		—
SCD	SIN LV	USA	Busulfan	Ongoing	—			
MLD	SIN LV	Italy (9)	Busulfan	Macrophages and microglia	HSCs	No symptoms or just milder, after the age of symptom onset. Ongoing	No	(86)
WAS	γ-RV	Germany (10)	Busulfan	Lymphoid cells, NK cells, and platelets	HSCs	9/10 with benefits	Yes	(169,170)
	SIN LV	UK, USA, France, Italy (4)	Busulfan +fludarabine			4 with benefits, ongoing	No	(48,171,172)
β-Thalassemia	SIN LV	USA, France (4)	Busulfan	Erythroid cells	HSCs	Therapeutic benefit. Ongoing	No	(173)

Disease	Vector	Patients	Conditioning regime	Target cells	Transduced cells	Outcome	Adverse effects	References
		USA	Busulfan			Ongoing		
FA	SIN LV	USA	None			Ongoing	—	
		Spain	None*			Ongoing	—	
X-ALD	SIN LV	London, France USA, France (4)	Busulfan and cyclophosphamide	Macrophages and microglia	HSCs	Therapeutic benefit. Ongoing	No	(174)

Table 1. Addition gene therapy attempts and their outcome.

Classically, viral vectors, based on γ-retroviruses (γ-RV) or lentiviruses (LV), have been widely used for this purpose because they ensure high transduction efficiency and long-term expression of the transgene. γ-RVs were widely used in the first gene therapy trials that started at the end of the 1990s (39). In diseases affecting the hematopoietic system, these vectors were first used for the treatment of two inmunodeficiencies: adenosine deaminase deficiency (ADA) and X-linked severe immunodeficiency or γ-chain immunodeficiency (X-SCID1) (40–42). In the case of ADA, γ-RV-transduced autologous stem cells have been used to treat 38 patients in three independent studies (39). Overall survival resulted in 100% at 3.5 years follow-up, and from these, 20 patients no longer required enzyme replacement therapy. *Ex vivo* gene transfer in ADA patients, by means of γ-RV, demonstrated to be efficient, as the engraftment of corrected cells was maintained over time and resulted in the improvement of the cellular and humoral immune response despite the mild conditioning applied (39). Similarly, the treatment of X-SCID1 was initiated in two different clinical trials. Similar to ADA gene therapy trials, the gene complementation of γ-chain with γ-RV was clearly efficient (42). Seventeen of the 20 treated patients showed a full or nearly full correction of the T-cell defect (39). However, whereas, in ADA patients treated by gene therapy with γ-RV, no adverse effects related with the genotoxicity of the procedure were observed, in X-SCID1 patients, 5 of the 20 treated patients suffered T-cell leukemia after 2.5 to 5 years after the treatment due to insertional mutagenesis (39). Viral insertion produces insertional mutagenesis and can induce the activation of neighboring genes, as proto-oncogenes, through enhancer/promoter sequences present in the retroviral long terminal repeats (LTRs). Both γ-RV and LV contain LTRs that are present at both the 5′ and 3′ ends of retroviral RNA genome and are required for the integration of the provirus into the host genome. Similar issues arose sometimes after the treatment of other immunodeficiencies as the Wiscott-Aldrich syndrome, in which restored expression of the WASP gene was achieved, but 4 of 10 treated patients were reported to suffer from leukemia or chronic granulomatosis disease (CGD) in which the appearance of myelodysplastic syndrome in 3 patients also proved to be the result of the up-regulation of genes such as *MECOM* and *PRDM16* in CGD patients (43–47). These severe adverse effects limited the application of gene therapy to patients. A strong effort of the scientific community was required to increase the safety characteristics of the vectors. The inactivation of the U3 region

in the 3′ end of the viral genome, leading to self-inactivating (SIN-LTRs) vectors (48), and the use of more physiological and/or specific promoters (49) were characteristics implemented in the new improved integrative vectors. Moreover, the analysis of insertion patterns demonstrated that γ-RV preferentially integrate near the transcriptional start sites and promoter regions (50), whereas this in LV occurs along transcriptionally active genes (51–53). Even so, some studies have reported that LV produce aberrant spliced transcript and deregulated expression in the integrated genes, although this phenomenon has not been reported in patients (54–56). Therefore, the SIN LTRs and the integration-site preferences of LV have been shown to substantially mitigate the insertional genotoxicity, making LV a reliable therapeutic option. In fact, a significant number of phase I/II clinical trials are currently being conducted with LV, which have reported remarkable efficacy and safety in immunodeficiencies, β-thalassemia, adrenoleukodystrophy, or metachromatic leukodystrophy (36). All these HSC-based gene therapy clinical trials have reported stable and high level of reconstitution of the hematopoiesis of the treated patients. Besides, there has been no report of adverse events related with LV, although the overall follow-up of some of these trials is still limited.

Nonetheless, the potential risks already observed with γ-RV could be still present. Future trials should overcome the efficient delivery of the new genetic information in the target cells, without altering or disrupting the host cell genome and preserving the characteristics of the transduced cells. The uses of LV that do not require integration or the precise control of the site where the genetic material is going to be inserted in the host genome (gene editing) are strategies that are nowadays being explored in deep. Important efforts are invested in this second approach that will overcome the majority of the potential adverse effects described above. However, additional challenges need to be overcome yet.

4. Gene editing strategies in HSCs

Gene editing describes the new technology able to accurately modify genes, either by knocking out these genes or by inserting or substituting specific sequences in a precise way. This new technology is based on promoting the action of endogenous DNA repair pathways at the target sequence without altering any other place in the genome. The main approach used for gene targeting is based on the natural HR DNA repair mechanism of the cell. Homologous Recombination (HR) is an accurate DNA repair mechanism that uses the sister chromatic as a template to repair double-stranded breaks (DSBs). HR-based gene therapy strategies started in 1980s using DNA donors flanked by long arms with homology to the target locus (57). However, the probability of inducing the specific insertion in the target site was low, around 10^{-6} (58), making this procedure only feasible for basic research. Trying to improve this low efficiency, several strategies have been addressed. The most important one is the development of engineered nucleases able to induce DSBs in the specific target site, with the consequent activation of the HR, and the gene edition in a precise place of the genome.

Although transfer of designed nucleases together with a donor template can increase the efficiency, HR is not as frequent as other DSB repair mechanisms, such as non-homologous

end-joining (NHEJ). This is an error-prone mechanism that binds the two DNA ends originated in the DSB, without the use of any template. Insertions and/or deletions (INDELS) can be introduced in the target site. HR process is more frequent during the S and G_2 phases of the cell cycle, so it takes place more frequently when cells are proliferating, and its permissiveness varies between cell types (59).

There are different applications based on these two DNA repair mechanisms. If NHEJ takes place, the generated INDELs could have a particular application for the stable disruption of specific target genes (59). When HR takes place, three strategies of gene editing can be envisaged:

- Gene correction: One or more bases from the mutated original strand are replaced by the integration of the corrected ones. This strategy is the one to be chosen when the objective is to introduce or repair point or small mutations. If the mutations causing the disease are not recurrent or are produced in different genes, it will be restricted to a limited number of patients.

- Knock-in: A partial cDNA of the gene of interest is introduced in its former locus. Usually, a splicing acceptor is located before the cDNA to anchor the endogenous splicing donor. Endogenous elements of the locus will control the expression of the endogenous/exogenous chimeric cDNA/gene. Necessary to maintain the endogenous regulation of transgene.

- Safe harbor integration: It consists on the insertion of a whole expression cassette (promoter, transgene and regulatory signals) in a concrete place of the genome. This approach could be used for mutations produced in the same gene and also for mutations produced in different genes.

5. Gene editing tools applicable to the gene editing of hematopoietic stem cells

Different systems have been developed to lead the genetic modification to a specific locus. These gene editing tools have been inspired by natural systems, which evolved over millions years to modify DNA, among which the capability of generating DSBs has been selected for different purposes including DNA repair or cell response against foreign DNA from pathogens. The proteins involved in DSBs act by the specific hydrolysis of the DNA and are known as nucleases. These nucleases have been described in all branches and life species. As long as these proteins have been described, they have been rapidly applied as tools for gene editing. Four different types of nucleases have been more widely applied to generate DSBs: homing endonucleases (also called meganucleases), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9.

5.1. Meganucleases

Meganucleases tend to be small proteins (<40 kDa) encoded by mobile inteins or introns. These families of proteins have evolved to target sequences (14–40 bp) in a specific way (60,61). In

nature, meganucleases propagate their own introns cleaving cognate intronless alleles and therefore producing HR. For that capability of moving its genetic sequence in a determined place, meganucleases are also called homing endonucleases. There have been described five families of meganucleases: GIY-YIG His-Cys box, HNH, LAGLIDADG, and PD-(D/E) XK. The largest family is known as LAGLIDADG, named after the consensus sequence of its catalytic motif LAGLIDADG. It is a diverse group of enzymes present in fungal and protozoan mitochondria, plants and algae chloroplasts, bacteria, and *Archaea*. This family is defined for having either one or two copies of LAGLIDADG motif acting therefore as separated monomers or homodimers, respectively. However, meganucleases are limited as gene editing tools due to the necessity of finding large palindromic DNA targets (62). Moreover, its design requires important bioinformatics, difficult protein engineering, and *in vitro* testing.

5.2. ZFNs

In 1996, Chandrasegaran et al. started to use hybrid nucleases which combined the cleavage domain of *Fok*I nuclease and the specificity of Cys_2His_2 zinc-finger protein domains, making possible the specific DNA cut in a sequence of more than 18 bp (63,64). *Fok*I is capable of generating DSB when dimerized, so to use it as a gene editing tool, two monomers must be designed and delivered separately. Designing the two monomers in inverted orientation improved the nuclease efficiency (65). The specific domain that binds to the DNA is formed by tandem repetitions of Cys_2His_2, which form the $\beta\beta\alpha$ structure folded for the presence of a Zn^{2+} ion. The α-helix recognizes a specific DNA triplet. Between 3 and 6 of these motifs, which are separated by some spacer nucleotides, are modularly assembled in each monomer. Between the recognition sites of both strains remains a small nontargeted region called "spacer" surrounded by the *Fok*I dimer (66–68). To avoid some off-target events already described (69,70), monomers of two different *Fok*I domains, only active when forming heterodimers, have been designed (71,72). Although different ZFN open-source libraries have been developed, the design still results complicated for nonspecialists (73,74). However, ZFNs have been the first described nucleases and are the only ones already in clinical trials (https://goo.gl/M0ZWoB).

5.3. TALEN

TALEN technology follows the same rationale as ZFN, the use of *Fok*I domain because of its nonspecific cleavage capability fused to a domain that confers DNA-binding specificity. As in the case of ZFN, the *Fok*I region requires dimerization to be active, so this tool also requires the use of two monomers (65,75). In TALEN, the specificity is provided for protein domains from TALE. TALE are bacterial proteins, first described in *Xanthomonas*, capable to modify the gene transcription of the plants that these bacteria parasite (76). This DNA-binding domain is formed by 10 to 30 repetitions of 30 residues. The 12th and 13th positions, the so-called repeat-variable di-residues (RVDs), are the polymorphism responsible of the specificity (77). Each RVD pair is able to recognize specifically a base pair (77–80). Knowing the code TALE-to-DNA (79), the designing has become a relatively easy task (81). Moreover, due to the long DNA sequences they recognize, they show the lowest off-target cleavage. On the contrary, their

highly repetitive structures make these sequences hard to package into viral vectors because of their tendency to recombine (82).

5.4. CRISPR/Cas9

The CRISPR/Cas9 system is the latest nuclease that has appeared in the gene editing tool arena. CRISPR/Cas9 is part of the adaptive immune system of bacteria and *Archaea*. In nature, this system cleaves foreign DNA to avoid cellular threats such as viral attacks (83,84). Up to 10 CRISPR/Cas9 systems have been described, but the type II CRISPR/Cas9 is the most used in gene engineering to generate DSBs for research.

This strategy is based in three elements: the Cas9 nuclease, the trans-activating CRISPR RNA (tracrRNA), and the CRISPR RNA (crRNA). Cas9 is a nuclease that produces DSBs, thanks to its two catalytic regions, Ruv and HNH. Cas9 recognizes the tertiary RNA structures that the duplex tracrRNA and crRNA form when they are bound to the DNA. The tracrRNA is a fix structure that binds Cas9, the catalytic element, to the crRNA, the element that gives the specificity to the target site. The crRNA is a 20-bp-long RNA strand complementary to the target region. The only restriction of the system is that the target sequences must be followed downstream for the 3-bp-long protospacer adjacent motif (PAMs), 5′-NGG-3′ (72). PAM motif is frequent along the DNA, so CRISPR/Cas9 system crRNA can be design against almost every DNA region. To facilitate its use, crRNA and tracrRNA have been combined in one single molecule called single guide RNA (gRNA) (85). Thus, only two elements must be delivered into the cells, the Cas9 and the gRNA, instead of three. Although controversial, some studies have reported a higher off-target activity than other nucleases (86). CRISPR/Cas9 system is developing fast and new Cas proteins recognizing different PAM sequences, having smaller protein sizes or even been able to cut in an overhang way have been described over the last year (87–89). Moreover, Cas9 protein modifications, as ZFN and TALE, have allowed not only the generation of DSB but also as function as DNA activators, DNA repressors, or DNA tracers (90).

6. Delivery of gene editing tools to HSCs: the main challenge

One of the most important steps to consider when performing a gene editing strategy is the delivery of the gene editing tools. In fact, nowadays this is probably the main bottleneck in gene therapy in general and definitely in the gene editing of HSCs (91).

The main objective of delivering the nucleases effectively in cells is to promote a hit-and-run activity of the enzyme that allows a short-term boost of expression that leads to a specific generation of a DSB in a short period of time to avoid toxicity and off-target activity due to the long-term expression of the nuclease. In case of performing a gene addition/gene correction strategy, we should also consider the delivery of the DNA molecule carrying the desired sequences to be integrated in the cell genome, the so-called donor DNA molecule. Taking all these facts into account, the delivery of gene editing tools can be classified in two different groups: viral and nonviral transfection methods (**Figure 2**).

Concerning the nonviral transfection methods, the gene editing tools can be delivered as plasmid DNA, *in vitro* transcribed mRNA, or purified proteins. The delivery of plasmid DNA is a widely used standard technique (92,93), as this molecule is easily engineered and easy to handle. Both gene editing tools and donor sequences can be transfected in this way. The donor sequence can be a linear donor sequence with less than 50 bp of homology (94), as well as single-stranded DNA oligonucleotides (95), especially when inducing mutations or corrections at the target site. Due to the negative charge of both plasmid DNA and the cell surface, the uptake of DNA into the cells is restricted, and the delivery needs to be supported by complexing DNA with chemicals such as calcium phosphate, coat lipids, or cationic polymers (92) or by subjecting the cells to an electric field that open pores in the nuclear and/or plasmatic membrane of the cells (electroporation). Another important factor to take into account is the target cell. In our case, the HSC is a hard-to-transfect cell. The only technique available nowadays to introduce these tools inside this cell type is nucleofection. Nucleofection is an electroporation-based transfection method that enables transfer of nucleic acids into difficult-to-transfect cells by applying a combination of electrical parameters with cell type-specific reagents. However, this process produces high cellular toxicity and even cell death depending on the plasmid size and the total amount of transfected DNA. Moreover, the delivery of plasmid DNA could mediate its random insertion into the genome of treated cells (96). The risk of random plasmid integration mainly depends on the plasmid quality, but it is also influenced by specific properties of the targeted cells, including the prevalence of DSBs and the status of their DNA repair pathways. Plasmid integration, although is a nonusual event, represents an obstacle for gene targeting approaches, as mentioned above for the more conventional retroviral vector delivery systems.

Alternative platforms to deliver nucleases have been explored, such as the transfection of *in vitro*-transcribed mRNA (97,98). The main advantage is that mRNA establishes a short-term boost of enzyme activity with low toxicity and no risk of integration (99). Thus *in vitro* transcribed mRNAs that encode ZFNs, TALENs, or Cas9 and gRNAs are the preferential forms in which these nucleases are nucleofected into HSCs. Another possibility is to deliver nucleases as proteins; this approach presents the same advantages as mRNA delivery, as they also act in a hit-and-run fashion. Genetic fusion of recombinant proteins to positively supercharged moieties favors their uptake by cellular internalization mechanisms (100,101). However, generation of high yields of soluble and active protein transduction domains (PTDs)-containing nucleases is difficult (102). An alternative strategy consists on the chemical conjugation of nucleases to PTDs for receptor-mediated endocytosis (103). Interestingly, due to the positive charge of their Cys_2-His_2 zinc-finger motifs, ZFNs display an intrinsic cell-penetrating capacity, which can lead to targeted mutagenesis (102). Other delivery options under investigation include protein transfection procedures such as electroporation (104). It has been successfully applied in the case of CRISPR/Cas9 nucleases, where HSCs have been efficiently edited by the direct nucleofection of the Cas9 protein/gRNA ribonucleoprotein complex (105). Chemical transfection agents are also being investigated for nucleases as proteins (106).

Apart from nucleofection, nonintegrating viral vectors, such as integrase-deficient LV vectors (IDLVs), adenovirus vectors (AdVs), and adeno-associated virus vectors (AAVs), also serve

as a robust source to facilitate the delivery of the genes encoding programmable nucleases both *in vitro* and *in vivo* in cell types that are sensitive to transfection such as HSCs (107). The decision whether to use one type or another relies on the size of the transgene to deliver and on the capability of the virus to transduce quiescent or diving cells.

AdVs are double-stranded DNA viruses capable of packaging up to 37 kb of DNA. However, the transduction capacity of these vectors is highly dependent on the cellular type. Owing to the large cargo size that can be accommodated in adenoviruses, they are an attractive platform for the delivery of programmable nucleases and donor DNA. They have been used in some gene targeting approaches to deliver nucleases in both fibroblasts and CD4+ T cells (108,109), but unfortunately they are not able to efficiently transduce HSCs.

AAVs are nonenveloped, single-stranded DNA viruses that replicate only in the presence of a helper virus, such as adenoviruses or herpes simplex viruses. Nowadays, they are the most widely used delivery system for *in vivo* gene therapy due their nonpathogenic behavior and their capability to overcome the host's preexisting immunity. The major disadvantage of AAVs is their low packaging capacity that is incompatible with some therapeutic gene sizes. ZFNs are the most compact nucleases, and sequences encoding ZFNs can be packaged into these vectors (110). In the case of the CRISPR/Cas9 tool, only one of the required elements can be packaged in an AAV. The *Streptococcus pyogenes* Cas9 can be packaged into an AAV, but the gRNA sequence should go in a different vector. An alternative to the limited packaging size of the AAV is the use of the *Staphylococcus aureus* miniCas9, which perfectly fits the AAV size with the gRNA sequence. However, the proper definition of the target sites for this miniCas9 is still being improved (87).

IDLVs are currently the preferred tool for transferring genes into HSCs, as they possess certain advantageous attributes compared to other vectors [reviewed in (38)]. For example, the preintegration complex of LV is actively translocated into the nucleus and thereby facilitates efficient transduction of a variety of nondividing cells. In contrast, other viral vectors depend on the dissolution of the nuclear membrane during mitosis for delivering their cargo into the target cell nucleus. Consequently, efficient transduction of HSCs can be achieved with IDLVs after a shorter incubation time *in vitro*, preserving to some extent the physiological nature of HSCs and their engraftment potential. IDLVs have successfully delivered ZFNs and homologous donor DNA (111). Unfortunately, IDLVs are incompatible with TALENs because highly homologous TALE repeats often lead to unwanted recombination in cells (112). Moreover, the production of IDLVs is still the major drawback. The high viral titers needed to efficiently transduce HSCs are difficult to get, limiting their potential use in many instances.

7. Evidences of gene editing in HSCs and future attempts

Since gene editing technology has started to be used, its applicability to HSCs is being explored. Either blood disease modeling or new gene therapy approaches have risen from the basics of gene edition. The development of genome editing technologies based on programmable nucleases has substantially improved the ability to make precise changes in the genomes of

the cells compared to small-molecule therapies. However, the gene editing advancement in hematopoiesis could not be understood without its combination with cell reprogramming technology, especially iPSCs. The correction by gene editing of patient-specific iPSC has facilitated the spreading of this technology, allowing the optimization of tools and techniques on this difficult but easily expandable cell type, which have been then applied to the more difficult to culture and transfect/transduce HSCs. On the contrary, the current clinical trials addressed to correct monogenic blood disease by viral vectors, retro and LV, have contributed to the useful knowledge about these diseases, different delivery systems, and their therapeutic potentials (Table 2).

The first evidence of gene editing in human hematopoietic progenitors was described in 2002 by Dr. Davis's group in sickle cell disease (SCD) HSCs (113). SCD is characterized by a single-point mutation in the seventh codon of the β-globin gene, which means that the site-specific correction of the mutation would allow for the production of normal hematopoietic cells. By DNA microinjection, chimeric oligonucleotides designed to direct a site-specific nucleotide exchange in the human β-globin gene were introduced into $CD34^+$ and Lin^- $CD38^-$ cells (purified from umbilical cord blood from healthy donors). Conversion rates of 10% to 15% were confirmed in Lin^- $CD38^-$ cells. These levels of correction are enough to correct the phenotype of the patients in this case, as 10% normal hematopoietic cells are sufficient to render patients free of SCD. However, human engraftment of edited HSCs in immunodeficient animals was not tested. The efficacy of gene editing in $CD34^+$ was later improved by the use of sequence-specific endonucleases. Dr. Naldini's group was able to identify gene edition in up to 80% hematopoietic progenitors after transducing cord blood $CD34^+$ with an IDLV carrying a puromycin-resistant gene together with ZFN targeting CCR5 locus and selecting them with puromycin (114). The first therapeutic application of gene editing was described 7 years later by the same group. Human HSCs were gene edited at the *IL2RG* locus, which is mutated in SCID-X1 patients, by an IDLV delivering a donor template to perform a knock-in strategy in combination with the electroporation of ZFN mRNAs against the same locus (115). Since the donor templates carried the GFP reporter cassette alone or together with the therapeutic *IL2RG* cDNA, the targeted human hematopoietic could be followed by GFP expression. GFP could be detected in up to 95% of immunodeficient mice transplanted with the human progenitors after their gene editing at short term after transplantation. However, the most significant achievement was the detection of the targeted human hematopoietic cells in 46% of the transplanted mice at long-term, which meant the targeting of the most primitive HSCs, the LT-HSCs, and opens up a real potential clinical use of gene editing in HSCs. More importantly, the IL2RG functionality was restored in T and NK cells derived from the hematopoietic progenitors edited at *IL2RG* locus. This is the first demonstration of the potential clinical use of gene edition to treat monogenic blood diseases.

Although the use of large donors to correct patient's hematopoietic progenitors through a knock-in or safe harbor approaches have got good preclinical data, its overall efficacy is still low to be applied in clinical trials. Alternatively, the use of single-stranded oligonucleotides (ssODNs) for HR has been also explored to perform gene correction due to their high efficacy and low toxicity. As Proof of concept, gene correction experiments using β-globin ssODNs as

donors to correct β-thalassemia were performed. Intracellular delivery was done using biodegradable nanoparticles loaded with triplex-forming peptide nucleic acids (PNAs). Although low levels of efficacy were reached (<1%), *in vivo* analysis showed engraftment of edited cells in NOD-SCID IL2rγ-null mice (116).

Recently, another approach was tested for the introduction of a mutation able to correct SCD (117). ZFNs were designed to specifically cleave at the β-globin locus with nonrelevant off-target effects and introduced in CD34⁺ cells (isolated from umbilical cord or peripheral blood) by electroporation. Simultaneously, the delivery of an IDLV or DNA oligonucleotide homologous donor template allows high levels of gene modification (10–20%) and engraftment in immunocompromised NSG mice.

The delivery of nucleases using AAV vectors has also demonstrated their feasibility for human HSCs gene editing. Their low toxicity and relatively easy manufacture, together with the ability of achieving high recombination frequencies with small homology regions (200 bp), make them feasible genetic instruments (118). Recent studies showed that AAV serotype 6 was efficient for the delivery of donor DNAs in human CD34⁺ Hematopoietic Progenitor Stem Cells (HPSCs) and can induce Homologous direct Repair (HDR) when used in combination with different nucleases (119). When AAV6 was used to deliver a GFP cassette flanked by arms homologous to CCR5 or AAVS1 locus, followed by electroporation with ZFN mRNA, HDR-mediated insertion at the CCR5 or AAVS1 locus was found in 17% to 26% of cells. Studies in immunodeficient mice showed that modified cells persisted in secondary transplant recipient animals, meaning that edited cells contained long-term HSCs.

Using a different gene editing approach, β-thalassemia correction has been achieved in a preclinical study without the need of a donor template (120). This clever approach was based in the knockout of the *BCL11A* gene using ZFN to generate INDELs in the mentioned gene. This gene regulates the transition from fetal globin (γ-globin) to an adult one (β-globin). Therefore, the disruption of *BCL11A* gene was able to ameliorate β-thalassemia due to increasing γ-globin and reducing mutated β-globin level.

The transfer of programmable nucleases into HSCs has been also developed as antiviral therapies by deleting genes encoding receptors essential to viral entry in the host cells, thus preventing the infection. The definitive cure of HIV or the achievement of efficient protection against this virus constitutes one of the biggest goals of modern medicine. Gene therapy can bring us closer to this purpose by the modification of *CCR5,* an HIV-1 coreceptor involved in the entrance of the viral particles in hematopoietic cells. The introduction by electroporation of specifically designed ZFNs against the *CCR5* gene attained 17% of allele disruption. This ZFN-treated HPSCs were capable of engraft in NOD-SCID IL2rγ-null mice and were able to produce a polyclonal multilineage progeny where *CCR5* disruption was maintained (121). Moreover, when mice transplanted with ZFN-modified cells were challenged with the HIV-1 virus, the animals were able to maintain the levels of CD4⁺ T cells throughout their tissues and showed lower levels of HIV-1 viral load compared to the control animals engrafted with nonedited HPSCs. In the same direction, other strategies such as the introduction of CCR5-Specific ZFNs or CCR5-MegaTALEN mRNAs into HPSCs using adenoviral vectors and electroporation, respectively, have demonstrated the potential of this (118,122).

Genome editing in HSCs has also been used to model cancer generation and development. For example, CD34$^+$ cells purified from human cord blood were capable of initiating leukemia in response to endogenous activated *MLL-AF9* or *MLL-ENL* oncogenes, generated by TALEN-mediated knock-in (123,124). These cells displayed altered *in vitro* growth potentials and induce acute leukemias following transplantation in immunocompromised mice at a mean latency of 16 weeks. Additionally, the phenotype, morphology, and molecular features of the induced leukemias were similar to patient leukemic blasts. Consequently, this strategy provides an experimental platform for prospective studies of mixed lineage leukemia (MLL). Similarly, the CRISPR/Cas9 system is also applicable to generate some cancer models. Translocations resembling those described in acute myeloid leukemia and Ewing's sarcoma (*RUNX1/ETO* and *EWS/FLI*, respectively) were generated in human cell lines and primary cells. Cas9 nuclease and the specific gRNA were introduced as plasmidic DNA by electroporation, reaching 4% efficiency in the generation of cancer-like translocations (125).

The application of gene editing to correct or study blood diseases is a growing field, where a rising number of publications exploring all the potential of this precise gene modification technology are being generated rapidly. We should be ready for the burst of gene editing in hematopoiesis.

8. Gene-edited HSCs goes to the clinic

A notable number of phase I/II gene therapy clinical trials have reported evidence of efficacy and safety for the treatment of various genetic diseases of the blood, including primary immunodeficiencies, leukodystrophies, thalassemia, and hemophilia. However, some difficulties are acting as a bottleneck in the clinical approach of gene editing tools. First, the delivery of genetic tools into hematopoietic progenitors must be improved. Second, an advance in the culture conditions is needed, given that gene-corrected cells must be present in large enough quantities to revert the condition and transmit the modification to their progeny. In this way, some compounds capable of preserving the "stemness" of CD34$^+$ HPSCs are nowadays being explored, such as SR-1 or UM171, with promising results (126).

Up to now, only one clinical trial worldwide is open to apply gene editing in human HSCs. The trial is addressing the safety of the transplantation of hematopoietic progenitors, gene edited for the *CCR5* disruption using specific ZFNs, into HIV-1-infected patients. These patients have undetectable virus but suboptimal CD4$^+$ cell levels. Phase 1 trial is ongoing and will provide first in human data about the use of gene editing tools (https://goo.gl/M0ZWoB).

9. Other alternative nonhematopoietic sources of HSCs and their edition

Gene edited autologous HSC transplantation (HSCT) approaches remain elusive because of the difficulty in the culture and expansion of long-term repopulating HSCs *ex vivo*, as these protocols still extend for long periods of time to achieve the delivery of the correcting tools

and the gene edition itself. An alternative therapeutic strategy is the use of patient-derived induced pluripotent stem cells (iPSCs) in which disease-causing mutations are corrected by gene targeting. The development of iPSC-based approach provides an unlimited source of subject-derived cells that bypasses the main limitation of HSCs-based protocols, as iPSCs possess properties of self-renewal and pluripotency that are similar to those of embryonic stem cells (127–129). Moreover, the availability of HSCs is compromised in some situations, and in this context, easily accessible cell sources, such as skin fibroblasts (130), keratinocytes (131), or peripheral blood mononuclear cells (132), constitute an advantage for gene editing protocols. These corrected iPSCs could then be differentiated into the desirable hematopoietic cell for transplantation into patients to treat the disease (133). Here are some of the most relevant attempts following this strategy.

Regarding erythroid lineage defects, efforts have focused on β-globin alterations causing β-thalassemia and SCD. Most of the works have reprogramed patient-derived cells by transduction of reprogramming factors, in the case of skin fibroblasts (134–137), or by nucle-ofection of amniotic fluid-derived cells (138,139). The gene editing strategy selected was a knock-in approach, and in the case of β-thalassemia patient-derived iPSCs, the delivery of TALENs (138,139) or CRISPR/Cas9 (135,139,140) by electroporation achieved specific gene targeting in human hemoglobin subunit β (HBB) locus, with variable HR rates. More precisely, in a direct comparison of CRISPR/Cas9 and TALEN, the first ones induced DSBs with greater efficiency than TALEN, but the second ones mediated a higher homologous gene targeting efficiency (139). In all the cases, the expression of HBB in colonies formed after clonogenic progenitor cell assays was comparable to endogenous β-globin allele, but the main challenge was the complete erythroid differentiation of the edited iPSCs, as embryonic and fetal but not adult hemoglobin was expressed (135,139). Singularly, Wang et al. reported that gene correction without assistant nucleases of iPSC reprogrammed from patient-derived fibroblasts and then differentiated to hematopoietic progenitors could differentiate *in vivo* and produce human β-globin in irradiated SCID mice, although the efficacy of correction was lower than 1% (134).

In the case of SCD, patient-derived fibroblasts were reprogrammed to iPSCs and subsequently edited by nucleofection of ZFN in a knock-in strategy. Recombination efficacy ranged from 1% to 10%, and as it happened in thalassemia studies, the vast majority of hemoglobin expression was of the fetal type (136,137).

The gene editing of iPSCs derived from peripheral blood mononuclear cells from pyruvate kinase deficiency (PKD) patients assisted by TALENs in a knock-in strategy rendered approx-imately 10% of efficacy in gene editing, obtaining high numbers of erythroid cells with a normalized ATP level and a normal metabolic function, providing an approach in which cells can undergo up to 2×10^4 -fold expansion to correct metabolic erythroid diseases (9).

In the case of primary immunodeficiencies, the access to BM and thymocyte samples from untreated patients with SCID is challenging, as these conditions are rare and infants typically presenting with life-threatening infections require urgent HSCT to survive (141). Different SCIDs have been addressed by gene editing of patient-derived iPSCs. Chang et al. demon-strated that locus-specific correction of the human JAK3 mutation by CRISPR/Cas9-enhanced

gene targeting of iPSCs derived from skin keratinocytes of SCID patients restored the differentiation potential of early T-cell progenitors. The strategy used was knock-in and efficacies ranged from 6% to 73 % of the analyzed clones when using one gRNA and from 0 to 100% of G418-resistant clones when using two guides. Corrected progenitors were capable of producing NK cells and mature T-cell populations expressing a broad TCR repertoire as well as all hematopoietic lineages. However, after transplantation in NSG mice, the differentiation to all hematopoietic lineages was not detectable (141).

Human mesenchymal cells from SCID-X1 patients were reprogrammed to iPSCs and afterwards corrected by HR assisted by TALENs in the *IL-2Rg* locus, following also a knock-in strategy, with an efficiency of 2.6% (142). They used a selection-free approach, as cells bearing a functional γ-chain show a positive selective advantage (143). The gene editing resulted in the rescue of T-cell precursors and mature NK cells after long-term differentiation *in vitro* (142). In ADA-SCID patients, the protocol was improved to perform reprogramming and gene targeting together in a one-step procedure that required only a single electroporation in an attempt to avoid cells to be in culture for several months, which is not compatible for patients for whom urgent medical intervention is imperative. They obtained 5% corrected iPSCs from skin biopsies of ADA-SCID patients after gene editing assisted by CRISPR/Cas9 and using single-stranded corrective oligonucleotide (ssODN) as therapeutic matrix, where the expression of corrected ADA mRNA was confirmed (144).

Some works have been performed in X-linked chronic granulomatous disease (X-CGD), being the selected strategy for gene editing the directed insertion of therapeutic cDNA in the safe harbor locus *AAVS1*. The selection of the promoter that drives the expression of the therapeutic gene seems to be quite important in CGD, as an inappropriate expression of the transgene in other cellular types, as the stem cell compartment, could lead to an exacerbate reactive oxygen species (ROS) production and engraftment failure (43,145). Merling et al. have developed a platform that combines the production of iPSCs derived from patients with the five genetic forms of CGD with the targeting of *AAVS1* safe harbor assisted by ZFNs, resulting in neutrophils and macrophages differentiated from the corrected iPSCs with a restored ROS production and antimicrobial function (146).

TALEN-assisted specific gene correction of *RUNX1* gene in iPSCs derived from dermal fibroblasts from a patient with familial platelet disorder resulted in the restoration of megakaryopoiesis and subsequent maturation of the corrected cells (147). Even in a DNA repair deficiency syndrome such as Fanconi anemia, with defects in homology-directed DNA repair, the wild-type cDNA of *FANCA* was targeted to *AAVS1*, assisted by ZFNs with an efficiency of gene targeting up to 4%, rendering disease-free hematopoietic progenitors (108).

Direct reprogramming, meaning the generation of hematopoietic cells or HSCs directly from nonhematopoietic cells, has been also assayed with only modest or even irreproducible results (148,149). No data are available related to the combination of gene correction with hematopoietic direct reprogramming.

Taking all these data into account, an iPSC-based approach would provide an unlimited source of patient-derived corrected cells from which hematopoietic cells could be derived continu-
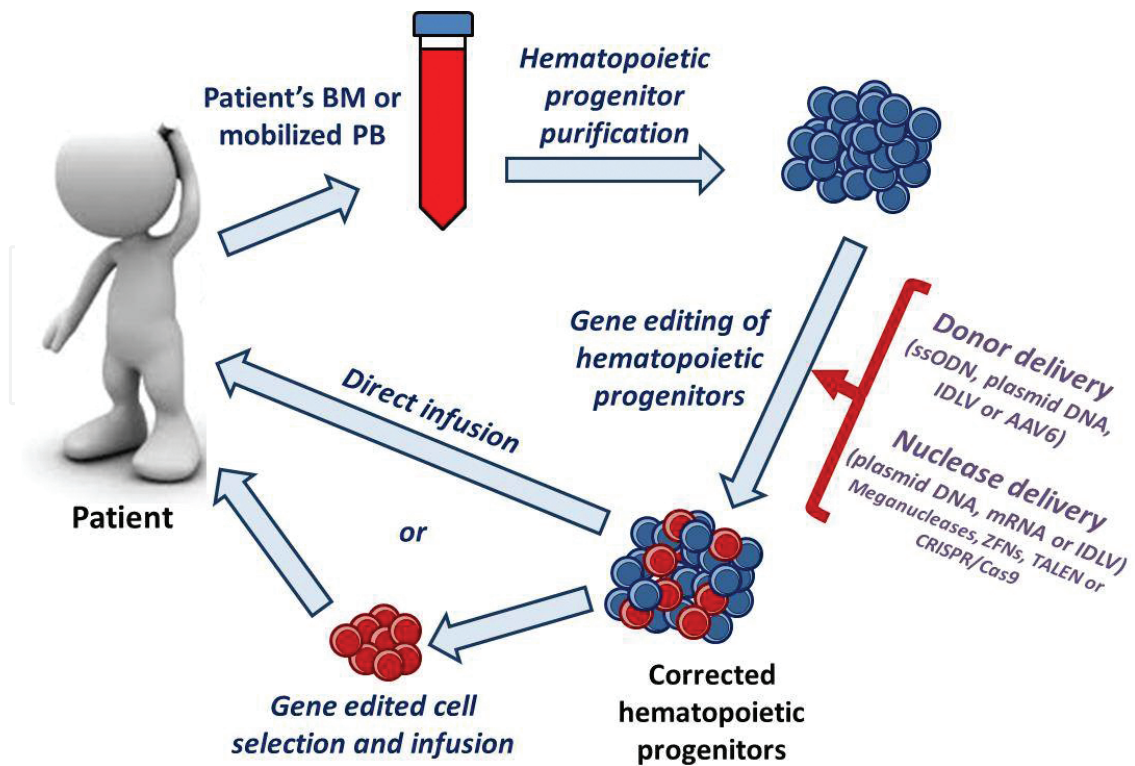
Figure 2. Potential strategies to follow in a HSC gene editing clinical protocol.

ously, and this approach could serve as a complementary approach to treat patients, for example, infusions of *in vitro*-derived autologous T cells to stabilize patients after HSCT (150). However, proper differentiations of the desirable cellular type, being the generation of real HSCs the major barrier, and an unsuitable engraftment of corrected cells remain the main pitfalls for the translation of this technology to the clinics.

10. Overall remarks

Although conventional gene therapy is becoming an alternative therapeutic option, being considered in some instances before allogeneic BM transplantation therapeutic option, the development of new tools and strategies to overcome the potential risks associated with the present ones is an already established challenge in the gene therapy field. Moreover, the precise correction of the mutation or mutations present in the patient's cells is the primary objective of any researcher committed to gene therapy. In this respect, gene editing is clearly an option to be explored and optimized. The gene editing field is constantly growing and changing. In the future, both hereditary and acquired diseases may benefit from gene editing strategies coupled with hematopoietic cell transplantation. As the conventional gene therapy of monogenic disease is the first and most successful clinical therapy involving genetic modification, the partnership between gene editing and hematopoietic progenitor transplantation will be one of the widest and effective gene therapies for these types of hematopoietic diseases.

Without any doubts, this traces the origin of a new era in cell and gene therapy and brings us closer to the aim of accurate site-specific gene editing in patients.

Cell source	Nuclease	Donor	Delivery system (nuclease/donor)	Strategy	Efficiency	Gene therapy	Gene edited HSC engraftment	Reference
Human UCB-CD34⁺ and Lin⁻ CD38⁻	No	Chimeric oligonucleotides	Microinjection	Gene correction	10–15%	Modeling point mutation of sickle-cell anemia	Not tested	(113)
Human UCB-CD34⁺	ZFN	CCR5 HA-PGK-GFP-CCR5 HA	IDLV/IDLV	Safe harbor (CCR5)	0.03–0.11%	Proof-of-concept	Not tested	(114)
Human mobilized PB-CD34⁺	No	β-Globin ssODN	PNAs	Gene correction	0.5–1%	Proof-of-concept	Not tested	(175)
Human mobilized PB-CD34⁺	No	CCR5 and β-globin ssODN	PNAs	Gene correction	0.43%	Proof-of- concept	Yes (in vivo gene editing of human hematopoietic cells engrafted in NSG)	(116)
Human UCB-CD34⁺ and BM-CD34⁺	ZFN	HA-PGK-GFP-HA and HA-IL2RG-cDNA-PGK-GFP-HA	mRNA electroporation/IDLV	Safe harbor (AAVS1) and knock-in	3–11%	SCID-X1 patient's HSC	Yes (NSG)	(115)
Human mobilized PB-CD34⁺	ZFN	No	mRNA electroporation	Knockout (BCL11A)	60–80%	β-Thalassemia	Not tested	(120)
Human BM-CD34⁺	ZFN	β-Globin donor IDLV or ssODN	mRNA electroporation/IDLV or ssODN electroporation	Gene correction	30–40%	Sickle-cell anemia	Yes (NSG)	(117)
Human mobilized PB-CD34⁺	ZFN	HA-GFP-HA or ssODN donor AAV6	mRNA electroporation/(CCR5 and AAVS1)	Safe harbor	17–26% or 19–43%	Proof-of-concept	Yes (NSG)	(119)

Cell source	Nuclease	Donor	Delivery system (nuclease/ donor)	Strategy	Efficiency	Gene therapy	Gene edited HSC engraftment	Reference
or fetal liver CD34+			AAV6 donor delivery					
Human mobilized PB-CD34+	ZFN	No	Plasmidic DNA electroporation	Knockout (CCR5)	17%	HIV protection	Yes (NSG)	(121)
Human mobilized PB-CD34+	ZFN	No	Adenoviral vector	Knockout (CCR5)	26–31%	HIV protection	Yes (NSG)	(176)
Human mobilized PB-CD34+	CRISPR/ Cas9	No	Plasmidic DNA electroporation	Knockout (B2M and CCR5)	26.8%	Hypoimmunogenic cells for transplantation and HIV protection	Yes (NSG)	(177)
Human mobilized PB-CD34+	ZFN/ TALEN	No	Adenoviral vector	Knockout (CCR5 and HS2-globin locus control region)	8.4–12%	HIV protection	Yes (NSG)	(122)
Human mobilized PB-CD34+	megaTAL	CCR5 HA-MND-GFP-CCR5 HA	mRNA electroporation/ AAV	Knockout (CCR5)	15–20%	HIV protection	Yes (NSG)	(178)
Human BM-CD34+	ZFN	No	Permeability mediated by multinuclear localization signal (multi-NLS ZFN)	Knockout (CCR5)	17%	HIV protection	Not tested	(179)
Human UCB-CD34+	CRISPR/ Cas9	No	Plasmidic DNA electroporation	Chromosomal translocation (RUNX1/ ETO)	0.96–4.07%	Cancer modeling	Not tested	(180)
Human UCB-CD34+	TALEN	MLL HA-cDNA(AF9, ENL2 or ENL7)-IRES-NeonGreen-MLL HA	Plasmidic DNA electroporation	Chromosomal translocation (MLL-AF9, MLL-ENL2, and MLL-ENL7)	9%	Cancer modeling	Yes (NSG)	(123)

Table 2. Present results in the gene editing of hematopoietic stem cells.

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