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REVIEW

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Advances in the gene therapy of monogenic blood cell diseases

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Abstract

Hematopoietic gene therapy has markedly progressed during the last 15 years both in terms of safety and efficacy. While a number of serious adverse events (SAE) were initially generated as a consequence of genotoxic insertions of gamma-retroviral vectors in the cell genome, no SAEs and excellent outcomes have been reported in patients infused with autologous hematopoietic stem cells (HSCs) transduced with self-inactivated lentiviral and gammaretroviral vectors. Advances in the field of HSC gene therapy have extended the number of monogenic diseases that can be treated with these approaches. Nowadays, evidence of clinical efficacy has been shown not only in primary immunodeficiencies, but also in other hematopoietic diseases, including beta-thalassemia and sickle cell anemia. In addition to the rapid progression of non-targeted gene therapies in the clinic, new approaches based on gene editing have been developed thanks to the discovery of designed nucleases and improved non-integrative vectors, which have markedly increased the efficacy and specificity of gene targeting to levels compatible with its clinical application. Based on advances achieved in the field of gene therapy, it can be envisaged that these therapies will soon be part of the therapeutic approaches used to treat life-threatening diseases of the hematopoietic system.

KEYWORDS
gene therapy, gene editing, hematopoietic stem cells, inherited diseases

1 | INTRODUCTION

While transplantation of allogeneic hematopoietic stem cells (HSCs) from healthy donors constitutes the standard therapy for patients with inherited hematopoietic diseases, the proportion of patients with HLA compatible donors is limited. Additionally, significant side effects mainly related to graft vs host disease (GVHD), infections and graft failure are associated to this therapeutic intervention. Due to these limitations, and because of advances in the development of gene therapy, the genetic correction of autologous HSCs is becoming an alternative therapeutic option to allogeneic transplantation in inherited hematopoietic diseases.

Based on the self-renewing and multi-potent properties of primitive HSCs, these rare bone marrow (BM) cells were considered an ideal target to correct genetic defects characteristic of inherited hematopoietic diseases. HSCs are responsible for the long-term generation of peripheral blood (PB) T- and B-lymphocytes, natural killer cells, monocytes, granulocytes, eosinophils, basophils, macrophages, erythrocytes and platelets.[1] Therefore, any monogenic disease associated with defects in blood cells could be potentially treated by means of the genetic correction of the HSCs.

Since the in vivo transduction of HSCs is a very inefficient process, current hematopoietic gene therapies are based on the collection and the ex vivo transduction of autologous HSCs with therapeutic vectors. Corrected cells are then reinfused into the patient, in most instances after sub-myeloablative or myeloablative conditioning to facilitate the engraftment of corrected cells. While

current trials of HSC gene therapy are based on non-targeted approaches with self-inactivated gamma-retroviral (RV) and lentiviral (LV) vectors, new approaches use more precise strategies based on gene editing (see schematic approaches of HSC gene therapy in Figure 1).

Despite the clinical efficacy of the first gene therapy trials conducted in patients with X-linked severe common immunodeficiency (SCID-X1)[2-4] and ADA-SCID,[5,6] and later on in chronic granulomatous disease (CGD)[7] and Wiscott-Aldrich (WAS) patients,[8] risks associated to the use of gamma-retroviral vectors (RVs) were observed in several patients whose HSCs were transduced with these vectors.[7-13] Nevertheless, the generation of self-inactivated lentiviral (SIN-LV) and gamma-retroviral vectors (SIN-RVs) has had an enormous impact on the clinical development of gene therapy since in addition to their clinical efficacy, no serious adverse events (SAEs) have been reported in patients treated with these new vectors.

Advances in the gene therapy of primary immunodeficiencies, β-hemoglobinopathies and bone marrow failure (BMF) syndromes will be described in this review. Additionally, the evolvement of HSC gene editing will be discussed due to its rapid progression.

2 | GENE THERAPY IN PRIMARY IMMUNODEFICIENCIES

Primary immunodeficiencies (PIDs) are a heterogeneous group of rare diseases associated with defects either in the number or the function of cells of the immune system. PIDs comprise a group of more than 300 genetic defects affecting the most important system responsible for protection against infections and cancer.[14,15] Depending on the specific PID, their incidence varies enormously.[16] Additionally, since the proper functioning of the immune system is required from the first weeks of life, PIDs frequently become life-threatening diseases that appear early in childhood. Clinical symptoms are heterogeneous, and are generally associated with a high rate of infections and even mortality. The only curative treatment for PIDs, besides ADA-SCID for which enzyme replacement has proven to be partially effective,[17] is hematopoietic stem cell transplantion (HSCT).[18] Outcomes of HSCT for PIDs have markedly improved after the first transplants were performed in WAS[19] and SCID[20] patients. Nevertheless, the possibility of finding a matched donor for patients with a severe PID is limited, in many instances due to the necessity of performing the transplant during the first months of life. Gene therapy was thus considered a good alternative for many of these patients, which led to a longer life expectancy of PID patients.[15]

Clinical trials in patients with SCID-X1[2-4,21] and ADA-SCID[5,6] clearly showed the benefit of gene therapy in PIDs. In both cases, RVs were used to facilitate the insertion of the therapeutic gene in patient HSCs. Unfortunately, 2 years after the initiation of the SCID-X1 trials SAEs consisting of lymphocytic leukemias were first observed in two patients due to insertional oncogenesis events. In both cases, RV integrations in the proximity of the *LMO2* proto-oncogene promoted the transactivation of this gene through the LTR (long terminal repeat) enhancer of the RV provirus.[9,10] Similar SAEs were then observed in other X1-SCID patients[3,4,11,12] and also in X-CGD[7,13] and WAS patients[22] (Table 1).

In contrast to the first ADA-SCID trials, in which no conditioning was used and where PEG-ADA was not stopped at the time of the infusion of tranduced $CD34^+$ cells,[34] in subsequent trials a moderate conditioning was used, and PEG-ADA was suspended prior to gene therapy. [6,25,27] These modifications markedly improved the efficacy of the therapeutic approach. Moreover, in contrast to observations in other PIDs, in none of the ADA-SCID-treated patients have SAEs been reported (Table 1), even though integration hotspots in different proto-oncogenes were identified. [5,6,25,27,34,35] Due to the similarities of RV backbones used in the ADA-SCID trial and in other trials where insertional oncogenesis events were generated, differences in either the therapeutic transgene or most probably in the nature of the

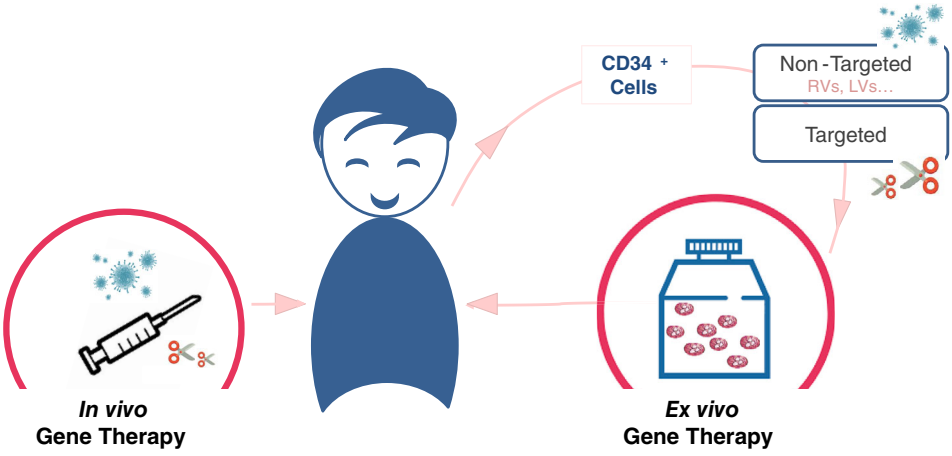

FIGURE 1 Gene therapy approaches for the treatment of monogenic blood cell diseases. Ex vivo gene therapy approaches are based on the collection patient's hematopoietic stem cells, followed by their genetic correction (either targeted or untargeted) and reinfusion of corrected cells in the patient. In vivo gene therapy approaches are based on the direct inoculation of viral or non-viral vectors in the patient, aiming the in situ genetic correction of affected cells

TABLE 1 Summary of gene therapy trials carried out with gamma-retroviral vectors (RV) and with self-inactivated lentiviral (SIN-LV) and gamma retroviral vectors (SIN-RV) trials in patients with primary immunodeficiencies

Disease	Vector	Vector promoter	Conditioning	Number of treated patients	Clinical efficacy	SAEs	Alive[§]	Reported follow-up (months)	References
X1-SCID	RV	LTR (MLV)	No	10	9	4	9	60-99	2,9-11,21
	RV	LTR (MLV)	No	10	9	1	10	54-107	3,4,12
	SIN-RV	EF1α	No	9	7	0	8	12-39	23
	SIN-LV	EF1α	RIC	5	5	0	5	6-30	24
ADA-SCID	RV	LTR (MLV)	RIC	18	15	0	18	28-161	5,6,25
	RV	RV LTR (MLV)	RIC	10	9	0	10	33-84	26
	RV	RV LTR (MLV)	RIC	6	4	0	6	24-84	27
	SIN-LV	EF1α	RIC	20	18	0	20	17–54	28
WAS	RV	RV LTR (MLV)	FC	10	9	7	8	25-81	8,22
	SIN-LV	WAS	FC	3	3	0	3	20-33	29
	SIN-LV	WAS	FC	7	6	0	6	9-42	30
CGD	RV	RV LTR (SFFV)	FC	2	0	2	1	26-45	7,13
	RV	RV LTR (MLV)	FC	2	0	0	2	36	31
	SIN-LV	Chimeric myeloid promoter	FC	7	7	0	7	ND	32,33

disease should account for the safety associated to ADA-SCID gene therapy. After the observation of the first SAEs in a number of PID patients, the gene therapy field rapidly progressed thanks to the development of safer therapeutic vectors. The generation of self-inactivated lentiviral vectors (SIN-LV)[36,37] and SIN-RV[38,39] soon showed the relevance of these new viral vectors. The safety of SIN-LVs was shown to be a consequence of both the inactivation of the enhancer activity of the HIV-1 LTRs—which dramatically reduced the transactivation potential of the provirus—and also of the integration properties of LVs, which in contrast to RVs do not preferentially target the transcription start sites. The possibility of using internal promoters facilitating the selective expression of the transgene in specific hematopoietic lineages constituted additional advantages of these new vectors over the first generation of RVs.[40-43]

SIN vectors, mainly SIN-LVs, rapidly became the preferred vectors for the treatment of PIDs. In the case of ADA-SCID and X1-SCID, the EF1α promoter was selected to drive expression of therapeutic transgenes.[24,44-46] In the case of X-CGD, a chimeric promoter consisting of the fusion of regulatory sequences of the c-fes and Cathepsin G genes was used to promote a preferential expression of gp91 in myeloid cells.[47] In the WAS trial,[29] the selected promoter included the regulatory region of the WAS promoter aiming at driving a physiological expression of WASP in PB cells[48,49] (see Table 1).

Thanks to the development of SIN-LV and SIN-RV, more than 100 patients with PIDs have been treated with these new vectors. Strikingly, no SAEs and excellent clinical outputs have been observed in these patients, suggesting that gene therapy will soon constitute a therapeutic alternative to HSCT for patients with PIDs.[50]

Advances in hematopoietic gene therapy have encouraged the development of new studies in other PIDs. This is the case of the leukocyte adhesion deficiency (LAD), which is a group of syndromes affecting leukocyte trafficking. Among them, LAD type I (LAD-I) is the most prevalent, affecting 1/1 000 000 births.[51] LAD-I is an autosomal recessive PID characterized by deficient β2 integrins expression.[52] These membrane glycoproteins are αβ heterodimers in which four different α subunits (CD11A, B, C and D proteins) dimerize with a common β subunit (CD18, encoded by the *ITGB2* gene). CD18 expression is thus required for normal leukocyte trafficking to infection sites. Therefore, the characteristic clinical feature of LAD-I patients is the increased number of infections that cannot be properly resolved. Two main phenotypes have been described in LAD-I. The severe phenotype, with less than 2% $CD18^+$ leukocytes in PB, is associated with life-threatening infections from the first days of life.[52-55] Patients with 2% to 30% of $CD18^+$ leukocytes have less severe clinical symptoms, including lower frequency of infections and a longer life expectancy.[51-54] As in other PIDs, the only curative treatment for these patients is HSCT from matched donors. Gene therapy thus appears as a very good alternative, mainly for severe LAD-I patients requiring an urgent cure very early in life. A first attempt to treat LAD-I patients by gene therapy used gibbon ape leukemia virus (GALV)-pseudotyped RVs. A very low and transient engraftment of corrected cells was observed in this trial, probably due to the absence of patient's conditioning[56] and to the fact that corrected LAD-I progenitor cells do not develop proliferative advantage. Recent experimental data[57] have raised expectations for gene therapy in these patients. Our studies showed the efficacy and safety of a gene therapy approach in an LAD-I mouse model using a SIN-LV in which a chimeric internal promoter[47]—already used in the gene therapy of X-CGD patients[58]—drives the expression of CD18. The Chim.hCD18-LV conferred phenotypic correction in mouse LAD-I leukocytes, which then expressed the heterodimer in their membrane and migrated to inflamed sites.[57]

Based on these experimental results it is expected that LAD-I will be added to the list of PIDs successfully treated by gene therapy.

3 | GENE THERAPY IN RED BLOOD CELL DISORDERS

Inherited red blood cell (RBC) disorders constitute a second and important group of inherited hematopoietic disorders that have been treated by gene therapy. This group includes hemoglobinopathies such as β-thalassemia (β-thal) and sickle cell disease (SCD), erythroid metabolic diseases like glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase deficiency (PKD), and erythroid membrane disorders like congenital dyserythropoietic anemia (CDA). Common symptoms include anemia and concomitant complications including jaundice, iron overload, extra-medullar hematopoiesis and gallstones, among others.

Hemoglobinopathies constitute the most prevalent RBC disorders. Approximately 5% of the world population carries a hemoglobin (Hb) alteration.[59] The incidence of hemoglobinopathies is even more frequent in areas where malaria is present because these pathologies, together with other RBC disorders such as PKD, confer resistance to the parasite infection.[60,61]

RBC disorders are caused by mutations in specific genes most of which have already been identified and cloned. These diseases can be cured by allogeneic HSCT, suggesting they are good candidates for hematopoietic gene therapy. As gene-corrected HSPCs from patients with RBC disorders do not develop advantage over diseased ones, full hematopoietic conditioning is required to eliminate endogenous HSCs, and to facilitate the engraftment of genetically corrected cells.[62,63]

In the case of hemoglobinopathies, protein levels of 10% to 30% are required to compensate the diseased phenotype of affected RBCs.[64] Additionally, the expression of globin proteins is tightly regulated and restricted to the erythroid lineage. Therefore, lineage-specific promoters were required in LVs designed for the treatment of hemoglobinopathies. Examples of these erythroid specific vectors are the BGI,[65] TNS9,[66] HPV569,[67] GLOBE,[68] and BB305[69] vectors.

After early gene therapy attempts to treat β-thal patients with RVs, safer SIN-LV with erythroid-specific promoters based on the globin locus control regions, were developed. These vectors showed their efficacy and safety in mouse models. A clinical trial was then developed in France with the BGI LV, showing clinical efficacy in β-thal patients.[65] A benign clone expansion was transiently observed due to the integration of the viral vector in the regulatory region of the *HGMA2* gene. Thereafter, the proportion of this clone in circulating nucleated cells declined to less than 10%,[65] while the patient remained with stable levels of the therapeutic hemoglobin and only required occasional transfusions.[70] Two other clinical trials have been conducted in the United States, Australia, France and Thailand using the BB305 vector. Most (92%) of the non-$\beta^0\beta^0$ patients remained transfusion-independent after a median follow up of 26 months. In the long-term, good although variable levels of gene correction were observed.[69] More severe $\beta^0\beta^0$ and severe $\beta^+\beta^+$ phenotypes showed a 73% reduction in annualized transfusion requirements. Importantly, vector integration studies showed a polyclonal reconstitution with no specific clonal dominance that could reflect a leukemic process due to insertional mutagenesis, thus revealing the safety of these gene therapy approaches[69] (Table 2).

A more recent trial was developed in Italy using the GLOBE LV. In this study, HSPCs were collected from PB after mobilization with G-CSF and plerixafor (an inhibitor of CXCR4/SDF1 chemokine signaling) and were infused intra-bone in patients treated with myeloablative conditioning with treosulfan plus thiotepa. This study showed rapid hematopoietic recovery with polyclonal multilineage engraftment of corrected cells, and a significant reduction and even discontinuation in the transfusion requirements.[72]

SCD is caused by the sickle mutation in the β-globin gene, which induces the polymerization of hemoglobin tetramers upon deoxygenation. These polymers generate the characteristic sickle shape of erythrocytes inducing SCD symptoms, such as hemolytic anemia and stroke.[80] As observed in β-thal, preclinical studies showed that LV-based gene therapy could be a therapeutic option for SCD. Vectors used for SCD were similar to those used for β-thal, although expressed anti-sickling globins, such as fetal γ-globin,[81] β^{T87Q}[82] or βAS3[83] mutants which inhibit deoxy-hemoglobin S (deoxy-HbS) polymerization. Clinical studies in SCD patients showed that the infusion of HSCs previously corrected with the BB305 vector expressing the β^{T87Q} anti-sickling globin resulted in transfusion independency for up to 2 years.[74] Multicenter studies have shown more difficulties in the development of efficient gene therapies in SCD patients, probably due to lower transduction efficiencies and poorer engraftment of transduced progenitors. Attempts to increase the expression of the fetal γ-globin gene have been conducted, either by overexpressing the γ-globin cDNA[70] or by inhibiting the expression of *BCL11A*, consequently activating the expression of the fetal γ-globin[84] (Table 2).

The second family of RBC disorders in which gene therapy has been used in preclinical models includes erythroid metabolic disorders—such as PKD (where the glycolysis energetic pathway is affected)—and erythropoietic protoporphyria (EPP), which affects *heme* metabolism.[85]

Pyruvate kinase (PK) is the metabolic enzyme that catalyzes the last step of glycolysis. Defective PK activity thus impairs cell metabolism in RBCs. Mutations in *PKLR*[86,87] cause pyruvate kinase deficiency (PKD), which constitutes the most frequent glycolytic enzymopathy. The prevalence of PKD has been estimated at 1 to 9 cases per 100 000 people in the Caucasian population (https://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=766). As in other RBC disorders, the main clinical symptom of PKD is hemolytic anemia of variable severity. Jaundice, cholelithiasis, splenomegaly, variable degrees of iron overload and reticulocytosis are additional complications caused by the disease. The mainstay of treatment consists of blood transfusions and iron chelation therapy. In severe cases, splenectomy may be required. However, all of these treatments are only palliative.[88]

Our laboratory showed the efficacy of RVs in correcting the disease in a PKD mouse model, and showed that human RPK expression

TABLE 2 Active gene therapy trials in patients with RBC syndromes

Disease	Vector	Promoter	Gene	Conditioning	Number	Efficacy	SAEs	Alive[c]	Reported FU (months)	References
β-Thalassemia	TNS9.3.55	Human β-globin promoter and arrayed erythroid-regulatory elements	β-Globin	Busulfan: 8 mg/kg	4	1	4[b]	4	8 y	65,66
	BB3025	Human β-globin locus control region	$\beta^{A\text{-}T87Q}$ globin	Busulfan: 12.8 mg/kg, pk-adjusted	22	20[a]	22[b]	22	36 mo	69,71
	GLOBE	Minimal promoter/enhancer element containing two hypersensitive sites from the β-globin locus control region	β-globin	Treosulfan 42 g/m^2 + Thiotepa 8 mg/kg	9	6	9	9	12 mo	72,73
Sickle Cell Disease	BB305	Human β-globin locus control region	$\beta^{A\text{-}T87Q}$ globin	Busulfan: 12.8 mg/kg, pk-adjusted	12	1	1[b]	12	15 mo	74,75
	βAS3-FB	Human β-globin locus control region	βAS3 globin	Busulfan: 12.8 mg/kg, pk-adjusted	7	4	7[b]	7	6 mo	76
	mLARβΔγV5	Human β-globin locus control region	γ-globin	Melphalan: 140 mg/m^2	2	2	2	2	12 mo	77
	LCR-shRNAmir	RNA polymerase (pol) III	BCL11A shRNAmir	Busulfan: 12.8 mg/kg, pk-adjusted	4	1 (pending data from the other three)	1[b] (pending data from the other three)	4	78 d	78,79

[a]Fifteen patients transfusion free, five with sustained production >2 g/dL of $\beta^{A\text{-}T87Q}$ hemoglobin total hemoglobin levels.
[b]Related to myeloablative conditioning.
[c]Patients alive at the end of the follow-up period.

was capable of fully correcting the PKD phenotype when more than 25% genetically corrected cells were transplanted.[89] A similar therapeutic threshold of corrected cells was reported in one PKD Basenji dog infused with foamy vector-corrected HSCs.[90] More recently, A therapeutic and clinically applicable LV that was as effective as the RV in curing the disease in PKD mice, which was generated in our laboratory, and showed a safe viral integration profile in mouse hematopoiesis.[91]

Erythropoietic protoporphyria (EPP) is an autosomal recessive disorder of the porphyrin metabolism caused by a decrease in the activity of ferrochelatase. This defect results in the accumulation of toxic PP in erythrocytes and liver, causing severe skin photosensitivity. The possibility of treating EPP by cell therapy was described in a mouse model of EPP (*Fech*$^{m1pas/+}$).[92-94] These authors reported a successful gene therapy treatment for EPP using an erythroid-specific SIN-LV, carrying the ferrochelatase cDNA under the control of the ankyrin-1 promoter linked to a mutated form of the *NF-E2/AP1* sequence motif of the *HS40* element,[95] although no clinical trials have been attempted so far in this disease.

4 | GENE THERAPY IN INHERITED BONE MARROW FAILURE SYNDROMES

Inherited bone marrow failure syndromes (IBMFS) comprise a wide range of diseases in which mutations in more than 80 different genes have been reported. IBMFS include Fanconi anemia (FA), dyskeratosis congenita (DC), Diamond-Blackfan anemia (DBA), Shwachman-Diamond (SD), severe congenital neutropenia (SCN) and congenital amegakaryocytic thrombocytopenia (CAT), all of which are associated with deficiencies in the production of blood cells.[96]

IBMFS are complex diseases with overlapping clinical manifestations, with BMF being a common feature and the main cause of mortality. IBMFS are produced as a consequence of mutations in genes involved in important biological functions, such as DNA repair, ribosome biogenesis or maintenance of the telomere length. As with PIDs, HSCT currently constitutes the only curative treatment of the BMF characteristic of these disorders.[97] Nevertheless, the difficulty in finding HLA-compatible donors, together with risks associated with pretransplant conditioning regimens and GVHD constitute the main limitations of HSCT in IBMFS.

Fanconi anemia (FA) is the most frequent IBMFS. Mutations in any of the 22 FA genes so far discovered account for the disease, with *FANCA* being the most frequently mutated FA gene (around 65% of FA patients worldwide have mutations in this gene).[98]

All FA proteins cooperate in a common pathway involved in the detection and repair of DNA inter-strand cross-links. The disruption of this key pathway leads to congenital abnormalities, BMF and cancer predisposition.[99] The inclusion of fludarabine (a potent immunosuppressive drug that does not cause DNA cross-linking) in conditioning regimens of FA patients markedly improved the outcome of transplanted patients.[100,101] Nevertheless, HSCT in FA still leads to side effects, such as increased incidence of squamous cell carcinomas, probably due to the use of genotoxic conditioning regimens and GVHD[102-104].

Gene therapy was thus considered a good alternative to HSCT for FA patients. However, difficulties in the development of FA gene therapy were derived from the low number of HSCs in these patients,[105] which limited the collection of clinically relevant numbers of HSCs either from the BM[106] or from G-CSF-mobilized PB[107,108] from these patients. Despite these difficulties, the observation of hematological improvements in FA mosaic patients suggested that the correction of a low number of HSCs could be sufficient to restore the hematopoiesis of these patients.[109-111] This phenomenon was considered a natural gene therapy process, which has been recently be reproduced by ex vivo gene therapy in Fanconi anemia subtype A (FA-A) CD34$^+$ cells transplanted into immunodeficient mice.[112] Although initial studies showed the efficiency of RVs to correct the phenotype of FA mouse HSCs and FA human hematopoietic progenitor cells (HPCs),[113-117] previous FA gene therapy trials with RVs failed to show the engraftment of corrected HSCs.[106,118,119] Different aspects may have limited the success of previous FA gene therapy trials in the clinic, including the low number of infused HSCs, the prolonged incubation period used to transduce these cells, or the absence of patient conditioning (see review in Reference 120).

The 24 hours-transduction of G-CSF/plerixafor mobilized FA-A CD34$^+$ cells under conditions that minimized oxidative and TNFα-induced damage facilitated the engraftment of corrected hematopoietic cells in immunodeficient mice.[112] Importantly, these cells showed a marked proliferative advantage over time, showing the feasibility of preserving the engraftment capacity of FA HSCs after gene therapy, which suggests that a similar proliferative advantage could take place in FA patients. Recently, two different trials using similar FANCA LVs have started in the United States and in Spain (see review in Reference 121, with preliminary results showing engraftment of corrected HSCs in the Spanish trial.[122]

Diamond-Blackfan anemia (DBA) is another rare congenital IBMFS that is clinically and also genetically very heterogeneous.[123] Approximately 55% of patients with DBA are associated with sporadic mutations in DBA genes.[124] In most instances, autosomal dominant mutations with incomplete penetrance have been characterized. So far, these mutations have been found in 20 out of the 80 genes encoding for human ribosomal proteins (RP). Mutations in *RPS19*[125] are observed in 25% of DBA patients. Data from the EuroDBA consortium showed that more than 90% of DBA patients are associated with mutations occurring in six DBA genes (*RPS19*, *RPL5*, *RPS26*, *RPL11*, *RPL35A* and *RPS24*).[126] In addition to mutations in RP genes, two non-RP genes have recently been reported in DBA patients: *GATA1* and *TSR2*.[127-130] While *GATA1* belongs to a family of transcription factors with an important role in the development of RBCs and platelets, *TSR2* codifies a protein involved in 20S pre-rRNA processing.

Allogeneic HSCT represents the only curative treatment for DBA patients. Nevertheless, side effects such as graft failure and GVHD limit the efficacy of HSCT in these patients. As reported in FA, the observation of mosaic DBA patients suggests the proliferative

advantage of reverted HSCs,[131,132] reinforcing the idea that gene therapy should constitute a relevant therapeutic strategy in DBA.

Previous experimental studies have shown that both RVs and LVs can correct the characteristic phenotype of DBA cells. In this respect, Hamaguchi *et al* showed proliferative defects in erythroid progenitors from patients with DBA and also that these defects could be ameliorated after complementation with RPS19-LVs.[133] Thereafter, the same group showed that the RV-mediated correction of $CD34^+$ cells from DBA patients favored the erythro-differentiation of these cells, and reported their repopulating ability in immunodeficient mice.[134]

Gene therapy studies conducted in a conditional *RPS19* knockdown mouse model with SIN-LVs showed that the ectopic expression of *RPS19* prevented the lethal BMF characteristic of these animals.[135] Because these studies used LVs harboring the strong SFFV promoter, subsequent studies were carried out with a more clinically relevant EFSα-RPS19 LV.[136] Based on preclinical gene therapy studies already conducted it is expected that gene therapy trials in patients with DBA will be developed in coming years.

Dyskeratosis congenita (DC) constitutes another IBMFS associated with mutations in any of the 15 genes related with the maintenance of telomere length.[137-140] Three inherited forms of the disease have been described: X-linked, autosomal dominant and autosomal recessive. The main manifestations of the disease are ungual dystrophy, leukoplakia, cutaneous hyperpigmentation, as well as BMF, which appears in 80% of DC patients before the age of 30.[141,142] In addition, patients can develop immunodeficiency, pulmonary fibrosis, kidney or liver failure and predisposition to develop myelodysplastic syndrome, acute myeloid leukemia and squamous cell carcinoma.[141] X-linked dyskeratosis congenita (X-DC) is one of the major variants of DC, and is caused by mutations in *DKC1*.[143] This gene encodes for dyskerin, a component of the telomerase complex.[144,145] In cells derived from patients with X-DC, telomerase activity is compromised as a consequence of the defect in dyskerin.[144]

Although allogeneic HSCT remains the only curative option for BMF of DC patients, the survival rate associated with the transplantation of these patients is still modest.[146,147] The development of safe and effective gene therapy approaches would thus prevent the main complications associated with allogeneic transplantation.

In vivo gene therapy studies aiming at the reversion of the BMF of *Trf*- and *Tert*-deficient mice have used adeno-associated viral vectors (AAV9) carrying a healthy copy of *Tert*.[148] Although AAVs can remain for long periods of time as episomal concatemers in non-dividing cells, in dividing cells AAV DNA is progressively diluted. Strikingly, in the study of Bar *et al*, BM cells expressed Tert up to 8 months after administration of the AAV.[148] Nevertheless, as total BM cells were used for assessing Tert expression, the possibility that this expression derive from non-dividing BM stromal cells has to be considered. In both models, a significant improvement of blood cell counts and elongation of telomere length were observed in PB and BM cells. The proof-of-concept provided by these studies suggested that AAV9 gene therapy approaches facilitating the expression of *Tert* might have a potential therapeutic effect for the BMF of DC patients. Nevertheless, safety issues related to the unregulated expression of TERT should be carefully considered before this therapeutic approach could be developed in the clinic.

Since X-DC patients with mutations in *DKC1* represent approximately 25% of DC patients, the complementation of this gene constitutes a relevant gene therapy approach in DC. However, different studies have shown that transfection of X-DC cells with *DKC1*-vectors does not correct the phenotype of these cells,[149-151] indicating that conventional gene therapy approaches with vectors expressing the *DKC1* gene may not constitute relevant strategies for the treatment of these patients. Strikingly, a small peptide of dyskerin—the GSE24.2 peptide—was shown to reactivate telomerase in X-DC cells.[150] The enhanced telomerase activity of GSE24.2-treated cells maintained the proliferation of these cells and decreased their oxidative stress, and also their DNA damage and senescence rate.[152] A smaller version of GSE24.2—the GSE4 peptide—has been developed more recently, showing similar efficacy.[153] Recent preliminary studies have suggested that the expression of this peptide in human cord blood $CD34^+$ cells may rescue the genetic defect in X-DC HSCs.[154]

5 | GENE EDITING: AN EMERGING GENE THERAPY APPROACH IN HSCS

Gene editing has experienced a major breakthrough during the last few years mainly due to advances in the design of nucleases capable of generating double strand breaks (DSBs) in the DNA, and thus of promoting homologous directed repair (HDR) in specific loci of the cell genome. Three main types of nucleases: ZFNs, TALEN and CRISPR/Cas9 have been used to target human HSCs. The efficacy of these nucleases to specifically target any region of the genome, together with the rapid development of CRISPR/Cas9 nucleases have spread the application of gene editing to treat many different pathologies using a variety of approaches that include correction of specific mutations [155], knock-in of therapeutic cDNAs into mutated loci [156], insertion of therapeutic cassettes into *safe harbor loci* [157], or inactivation of regulatory sequences inhibiting the expression of specific genes to compensate the loss of function of mutated genes[158,159] (see schematic representations of different gene editing approaches in Figure 2). All these advances are rapidly moving gene editing into the clinic for the treatment of hematopoietic inherited diseases[160-162].

Different approaches have been explored to facilitate the delivery of nucleases and donor sequences into human HSCs. Electroporation, currently constitutes one of the most frequent methods used for the delivery of nucleases. On the other hand, transduction of HSCs with non-integrative viral vectors constitutes the most efficient method for the delivery of the donor constructs. ZFNs and integrase-defective lentiviral vectors (IDLVs) were successfully used for the editing of human HSCs.[163] Although gene-editing efficiencies were markedly reduced in primitive HSCs as compared to the bulk of $CD34^+$ cells, this study opened the possibility of using gene editing strategies for the treatment of hematological disorders. The same study showed the feasibility of correcting BM HSCs from SCID-X1 patients by means of the insertion of exons 5 to 8 of *IL2RG* cDNA in the endogenous *IL2RG*

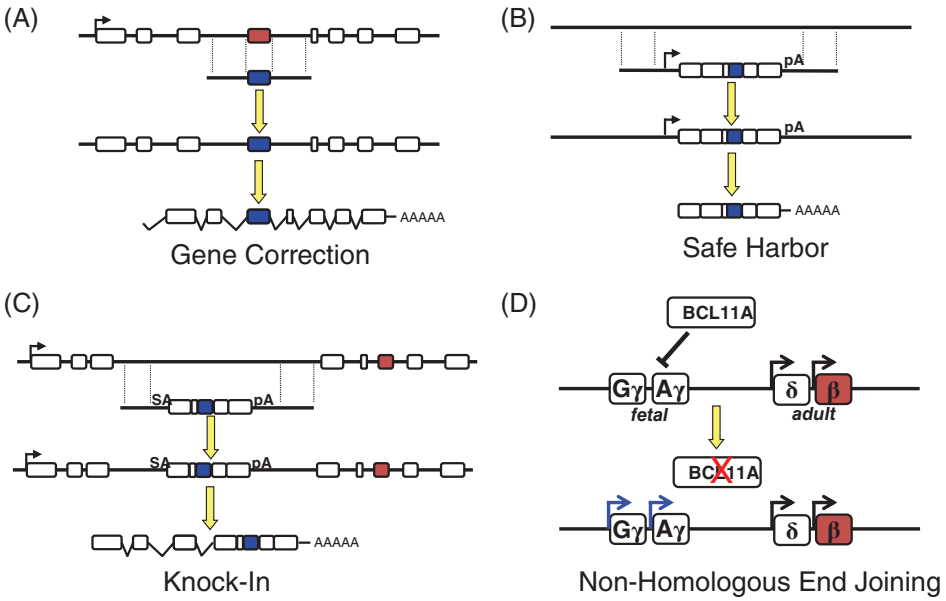

FIGURE 2 Illustration of different gene editing strategies. A, Gene correction: the mutation (red) is replaced by a wild-type sequence (blue). B, Insertion in *safe harbor* loci: an expression cassette is inserted in specific *safe harbor loci.* The therapeutic cassette includes a constitutive promoter, the therapeutic cDNA and a polyA (pA) sequence. C, Knock-in in homologous genes: the therapeutic cDNA is inserted in the mutated locus, together with a splicing acceptor (SA) and a polyA (pA) sequence. D, Non-homologous end joining (NHEJ)-based editing: generation of insertions and deletions (indels) in the targeted gene (eg, the generation of Indels in *BCL11A* allows the expression of fetal globins). β-globin locus is shown, where the expression of fetal globin genes (Gγ and Aγ) are regulated by BCL11A. Mutated β-globin gene is shown as a red box

gene. A more recent study has shown gene correction of SCID-X1 HSCs using a CRISPR-Cas9/AAV6-based strategy for the integration of the *IL2RG* cDNA into the endogenous start codon of this gene.[164] Additional studies confirmed the possibility of editing SCD HSCs by the use of ZFNs combined with IDLVs to deliver the therapeutic cDNA into the β-globin gene.[165] Our group also showed the phenotypic correction of HSPCs from FA patients through the specific integration of *FANCA* in the *AAVS1* locus using a similar combination of ZFN mRNAs and therapeutic donor IDLVs.[157]

More recently, the use of AAVs carrying donor sequences has been explored to facilitate the editing of primitive HSCs. AAVs are non-integrative vectors that can carry either a single or a double stranded DNA as donor templates for HDR. Among the different AAV serotypes, AAV6 is particularly efficient for transducing HSCs.[166,167] Thus, AAV6 outperformed gene editing efficiencies as compared to IDLVs, facilitating efficacies of gene editing between 20% and 40% when combined with appropriately designed nucleases.[156,168]

An important advance in the field of gene editing was the discovery of CRISPR/Cas9 nucleases and the observation that this nuclease system could be efficiently delivered in HSPCs by the use of ribonucleoprotein (RNP) complexes.[169] These RNPs have been implemented with the design of gRNAs aiming at increasing their stability,[170] thus achieving high HDR efficiencies in HSPCs.[155,171] These new RNPs together with the use of AAV6 for the delivery of donor templates are thus approaching the field of gene editing to the treatment of patients with hematological disorders.[156,172,173] To reduce the complexity of gene editing—for example, by avoiding handling of viral vectors—the co-delivery of nucleases together with ssODN donors has been used to correct specific mutations, including the SCD mutation in HSCs.[155,165]

Since non-homologous end joining (NHEJ) is the most efficient mechanism for the repair of DSBs, particularly in non-dividing cells,[174,175] this strategy has been used in the first FDA approved gene editing trials with autologous HSCs. This trial aims at the treatment of HIV infection using ZFNs cleaving the *CCR5* locus, that encodes a HIV receptor.[176] In the field of hemoglobinopathies, the knock-out of the *BCL11A* gene (Figure 2D)—a repressor of fetal globin[177]—facilitated the reexpression of fetal globin in adults cells.[178] This strategy will soon be used in gene editing trials of β-thalassemia[160,161] and SCD[162].

Improvements in the gene-editing field are facilitating its implementation as gene therapy approaches to treat hematopoietic-inherited diseases. The most concerning issue of gene-editing technologies are the off-target effects caused by engineered nucleases. In most instances, only a few in silico predicted off-target sites are frequently analyzed, although deep off-target analyses will be required in clinical gene-editing trials. Different approaches, such as GUIDE-seq[179] or CIRCLE-seq[180] have been developed to facilitate the identification of off-targets in the human genome. Furthermore, different refinements in gene-editing strategies have been established to reduce the generation of off-targets, including paired Cas9 nickases,[181] or high-fidelity Cas9 nucleases.[172]

Gene editing is a growing field that constitutes the cutting edge for the treatment of hematopoietic inherited diseases. New strategies to correct these disorders based on gene editing will indeed appear, including the use of base editors to correct point mutations, or the in vivo gene editing of mutated cells.

6 | PERSPECTIVES OF HEMATOPOIETIC GENE THERAPY

As deduced from recent gene therapy trials conducted with self-inactivated RV and LVs, the clinical efficacy and safety associated to the use of these new vectors is now evident. As has happened with the approval of *Strimvelis* for the treatment of ADA-SCID patients, several new approvals of medicinal products based on the genetic correction of HSCs will appear in upcoming years. Discussions about the efficacy and safety of gene therapy are rapidly moving toward additional questions related to the cost of these new therapies and the procedures that should be used to facilitate the spread of these new therapies to patients.[182] Shall patients travel to specialized gene therapy institutions, or shall these new medicinal products be delivered under appropriate conditions to facilitate its application in local institutions? All these aspects are open questions that need to be carefully evaluated for the appropriate application of gene therapy in the clinic. Additionally, practical procedures should be developed to facilitate that these new therapies could be efficiently transferred from academic institutions to pharmaceutical and biotech companies capable of manufacturing these medicinal products at a large scale and under highly controlled manufacturing conditions.

While conventional viral gene therapy is becoming an established therapeutic option in different disorders, advanced gene therapies such as gene editing are rapidly emerging. Thus, new challenges will continuously appear, such as the necessity of limiting potential side effects related to the off-target activity of designed nucleases.

Based on advances achieved in the field of hematopoietic gene therapy there is no doubt that this new therapeutic modality will constitute part of the therapeutic arsenal for the treatment of complex and life-threatening diseases.

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CONFLICT OF INTEREST

The Hematopoietic Innovative Therapies Division at CIEMAT receives funding and has licensed therapeutic lentiviral vectors to Rocket Pharmaceuticals Inc. JAB and JCS are consultants for Rocket Pharmaceuticals Inc. Authors are inventors on patents on lentiviral vectors filled by CIEMAT, CIBERER and FJD and may be entitled to receive financial benefits from the licensing of such patents.

DATA ACCESSIBILITY

Does not apply.

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