Chapter 21

Gene Therapy for Erythroid Metabolic Inherited Diseases

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Additional information is available at the end of the chapter

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1. Introduction

Gene therapy is becoming a powerful tool to treat genetic diseases. Clinical trials performed during last two decades have demonstrated its usefulness in the treatment of several genetic diseases [1] but also the need to improve vector delivery, expression and safety [2]. New vectors should reduce genotoxicity (genomic alteration due to vector integration), immunogenicity (immune response to gene delivery vectors and/or trangenes) and cytotoxicity (induced by ectopic expression and/or overexpression of the transgene).

In mature erythrocytes, most metabolic needs are covered by glycolysis, oxidative pentose phosphate pathway and glutathione cycle. Hereditary enzyme deficiencies of all these pathways have been identified, being most of them associated with chronic non-spherocytic hemolitic anemia (CNSHA). Hereditary hemolytic anemia exhibits a high molecular heterogeneity with a wide number of different mutations involved in the structural genes of nearly all affected enzymes. Deficiency in metabolic enzymes impairs energy balance in the erythrocytes, with or without changes in oxygen affinity of hemoglobin and delivery to the tissues. Despite of having a better understanding of their molecular basis, definitive curative therapy for Red Blood Cells (RBC) enzyme defects still remains undeveloped.

Conventional bone marrow transplantation allows the generation of donor-derived functional hematopoietic cells of all lineages in the host, and represents the standard of care or at least a valid therapeutic option for many inherited diseases [3]. However, complications associated to allogeneic transplantation can be as severe as the enzymatic deficiency. The recessive inheriting trait of most of these metabolic diseases and the confined enzymatic defect to the hematopoietic/

erythropoietic system, make them suitable diseases to be treated by gene therapy. Correction by gene therapy requires the stable transfer of a functional gene into the autologous self-renewing Hematopoietic stem cells (HSCs) and their mature progeny. Autologous BM transplantation of genetically corrected cells shows several advantages over the allogeneic procedure. First, it overcomes the limitation of human leukocyte antigen (HLA)-compatible donor availability, so it can be applied to every patient. Second, the reduction of morbidity and mortality associated with the transplant procedure, as there is no risk of graft versus host disease (GvHD) and consequently no need for post-transplant immunosuppression.

To date, gene therapy approaches for the treatment of inherited metabolic deficiencies are still limited, mainly because of the frequent lack of selective advantage of genetically corrected cells. This implies that high levels of transgene expression are required, as well as an efficient transduction of HSCs. This requirement have already been described in different RBC diseases as in the erytropoietic protoporphyria (EPP) [4] caused by the deficiency of the last enzyme of the heme biosintesis pathway or in the piruvate kinase deficiency (PKD) [3], where there is an impairment in the final yield of ATP in RBC. Additionally, some RBC pathologies require switching on expression of the transgene at only the proper stage of differentiation, which represents another challenge in the development of new gene therapy protocols.

2. Gene therapy attempts for inherited metabolic diseases of erythrocytes

Although more than 14 metabolic deficiencies have been identified causing CNSHA, approaches of gene therapy have been done only in a few of them (Table 1). Below, we are including a short description of the different diseases and the attempts addressed.

Among glycolytic defects causing CNSHA, Glucose 6-phosphate dehydrogenase (G6PD) deficiency is the most common genetic disease. More than 400 million people are affected world wide, showing a vast variability of clinical features. G6PD catalyzes the first reaction of the pentose phosphate pathway, in which Glucose 6-phosphate (G6P) is oxidized and Nicotinamide adenine dinucleotide phosphate is reduced (NADPH) resulting in decarboxylation of CO_2 and pentose phosphate. G6PD plays a central role in the cellular physiology as it is the major source of NADPH, required by many essential cellular systems including the antioxidant pathways, nitric oxide synthase, NADPH oxidase, cytochrome p450 system and others. Indeed, G6PD is essential for cell survival. *G6PD* is a 20 kb X-linked gene that maps to the Xq28 region, consisting of 13 exons and 12 introns, which encode a 514 amino acids protein with ubiquitous expression. More than 100 missense mutations in the *G6PD* gene have been identified [14], being most of them single-point mutations causing an amino acid substitution. Frequently, these mutations cause mild symptoms or no disease, except when patients are challenged by increased oxidative stress or fava beans. However, some mutations provoke severe instability of the G6PD and, as a result, lifelong CNSHA with a variable severity [15,16]. Through genetic studies it has been observed that severe clinical manifestations appear preferentially in exons 7, 10 and 11. As *G6PD* is X-linked, the defect is fully expressed in affected males (hemizygotes who inherit the mutation only from the

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mother), whereas in homozygous females the mutations are transmitted from both parents. Thereby, female heterozygotes represent a red blood cell mosaic population, causing a wide range clinical picture.

Disease	Gene	Chrom.	Inheritance	Other symptoms	Bone Marrow Transplantation	Gene Therapy
Glucose-6 Phosphate Dehydrogenase Deficiency (G6PD)	*G6PD*	Xq28	X-linked	jaundice, spleno- and hepatomegaly, hemoglobinuria, leukocyte disfunction, and susceptibility to infections		D: C57BL/6 mice P: Transduction of 5-FU treated BM cells with MMLV-hG6PD or MPSV-hG6PD vectors and subsequent transplantation. R: lethally irradiated C57BL/6 mice [5]
Pyruvate Kinase Deficiency (PKD)	*PKLR*	1q21	A.R	Reticulocytosis, splenomegaly, *hidrops foetalis*, and death in neonatal period	D: normal CBA/N$^{+/+}$ mice + 5FU R: CBA Pk-1^{slc}/ PK-1^{slc} mice C: minimal (100 or 400 cGy) [6]	D: WT mice P: Transduction of 5-FU treated BM cells with pMNSM-hLPK retroviral vector and subsequent transplantation R: lethally irradiated mice [7]
					D: normal CBA/N$^{+/+}$ mice R: CBA Pk-1^{slc}/ PK-1^{slc} mice C: no conditioning [8]	D: CBA PK-1^{slc}/PK-1^{slc} mice P: Transgenic rescue using the µLCR-PKLR-hRPK construct [9]
					D: normal Basenji dogs R: PKD Basenji dogs C: sublethal dose (200 cGy) + mycophenolate memofetil + cyclosporine [10]	D: WT mice P: Transduction of Lin^{-}Sca1^{+} BM cells with a MSFV-hRPK retroviral vector and subsequent transplantation R: lethally irradiated WT mice [11]
					D: HLA-identical sister R: PKD severe patient C: busulfan + cyclophosphamide [12]	D: AcB55 mice P: Transduction of Lin^{-}Sca1^{+} BM cells with a MSFV-hRPK retroviral vector and subsequent transplantation R: lethally irradiated AcB55 mice [13]
Glucose Phosphate Isomerase Deficiency (GPI)	*GPI*	19q13.1	A.R	neuromuscular disturbances		
Triose Phosphate Isomerase Deficiency (TPI)	*TPI1*	12p13	A.R	neuromuscular disorders, mental retardation, frecuent infections and death *in utero*		
Hexokinase Deficiency (HK)	*HK1*	10q22	A.R	defects in platelets		
Phosphofructokinase Deficiency (PFK)	*PFKL*	21q22.3	A.R	myopathy, storage disease type VII		
Bisphosphoglycerate Mutase Deficiency (BPGM)	*BPGM*	7q31-q34	A.R	erythrocytosis		
Glutathion Synthetase Deficiency (GSD)	*GSS*	20q11.2	A.R	5-oxoprolinuria, metabolic acidosis, central nervous system impairment		

A.R, autosomic recessive; D, donor; R, receptor; C, conditioning; P, protocol

Table 1. Most Common Erythroid Metabolic Inherited Diseases. BM transplantation and gene therapy approaches

Patients with CNSHA suffer anemia and jaundice, but often tolerate their condition well. However, G6PD variants with low activity are related with alterations in the erythrocyte membrane facilitating its breakdown and causing intravasal hemolysis. These symptoms are often accompanied by spleno- and hepatomegaly and hemoglobinuria. Besides, leukocyte dysfunctions caused by lower concentration of NADPH appear when G6PD activity is below 5% of the normal activity, leading to an immune depression [17]. Vives *et al.* and other groups have also observed an increased susceptibility to infections [18,19].

Preclinical work from Rovira et al demonstrates that *hG6PD* gene transfer into HSCs may be a viable strategy for the treatment of severe G6PD deficiency [5]. Through the transplantation of pluripotent hematopetic stem cells transduced with γ-retroviral vectors carrying the wild type human G6PD cDNA, they achieved a stable and lifelong expression of hG6PD in all the hematopoietic tissues of primary and secondary receptor mice. In this study, transgene expression was driven by the 3' LTR from either the Moloney murine leukemia virus (MMLV) or the myeloproliferative sarcoma virus (MPSV), obtaining an efficient transduction in murine hematopoietic progenitors. The corrected cells were then injected into lethally irradiated syngeneic mice, increasing 2-fold the enzyme activity in peripheral blood cells in comparison with non-transplanted control mice. Long-term hG6PD expression derived from the vector was also observed, which was similar to that of the endogenous enzyme activity. Similar expression was detected in RBC and in White Blood Cells (WBC) in different hematopoietic organs, as expected due to the use of a viral ubiquitous promoter. These results support gene therapy as a suitable strategy for the treatment of severe CNSHA due to G6PD deficiency. Additionally, they also demonstrated the efficacy of this gene therapy vector in human embryonic stem cells (hESC) in which the *G6PD* gene had been inactivated by targeted homologous recombination, which implies the potential application of gene therapy to G6PD hESCs. Moreover, although a selective advantage in favor of G6PD corrected cells has not been reported because the mice used showed normal G6PD activity, Rovira et al observed a strong selection after transduction of G6PD-deficient ES cells with their vectors. In this regard, the development of G6PD deficient mouse models would be a valuable tool to test new protocols. Furthermore, the mouse strain recently developed by Hay Ko et al may be useful, although it does not reproduce all the features of the human G6PD-deficiency [20].

Pyruvate kinase deficiency (PKD), the second most frequent abnormality of glycolysis causing CNSHA, has also been proposed as a potential disease to be treated by gene therapy. Pyruvate kinase (PK) catalyzes the second ATP generation reaction of the glycolysis pathway by converting phosphoenolpyruvate (PEP) into pyruvate, yielding nearly 50% of the total ATP production in red blood cells. PK plays a crucial role in erythrocyte metabolism, since mature RBC are absolutely dependent on the ATP generated by glycolysis, giving the loss of mitochondria, nucleus and endoplasmic reticulum in their mature state. RPK is therefore necessary for maintaining cell integrity and function. Reduced levels of erythrocyte Pyruvate kinase (RPK) lead to an accumulation of glycolytic intermediates that ultimately shortens the life span of mature RBC by metabolic block [21]. Four tissue-specific isoenzymes of PK (M1, M2, R and L) encoded by two different genes (*PK-M* and *PK-LR*) have been identified in humans [22]. The *PK-LR* gene, located on chromosome 1 (1q21) [23] encodes for both LPK (expressed in liver, renal cortex and small

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intestine) and RPK (restricted to erythrocytes) through the use of alternative promoters [24]. PK-M1 is expressed in adult nomal tissue, like brain or muscle. The PK-M2 isoform is typically expressed in proliferating tissues like fetal, tumoral and several other adult tissues [25] and during the maturation of the erythroblasts, gradually decreases, giving rise to the RPK isoform.

The codifying region of *PK-LR* gene is split into twelve exons, ten of which are shared by the two isoforms, while exons 1 and 2 are specific for the erythrocyte and the hepatic isoenzyme respectively [26]. However, clinical symptoms caused by *PK-LR* mutations are confined to RBC because the hepatic deficiency is usually compensated by the persistent enzyme synthesis in hepatocytes [27]. To date, more than 150 different mutations in the *PK-LR* have been associated with CNSHA, being most of them missense mutations, splicing and codon stop. Only two variants, -72 G and -83 C, have been identified in the promoter regions so far [26,27]. Molecular studies indicate that severe syndrome is commonly associated with disruptive mutations and missense mutations involving the active site or protein stability [28].

PK deficiency is transmitted as an autosomal recessive trait and although its global incidence is still unknown, it has been estimated in 1:20000 in the general caucasian population [29]. Clinical symptoms appear in homozygous and compound heterozygous patients, which lead to a very variable clinical picture, ranging from mild or fully compensated forms to life-treating neonatal anemia necessitating exchange transfusions and subsequent continuous support [28]. Pathological manifestations are usually observed when enzyme activity falls below 25% of normal PK activity [30], and severe disease has been associated with a high degree of reticulocytosis [31]. *Hydrops foetalis* and death in the neonatal period have also been reported in rare cases [32,33]. PK deficiency treatment is based on supportive measures since no specific therapy for severe cases is available to date. Periodic cell transfusions may be required in severe anemic cases, often impairing their quality of life. Splenectomy can be clinically useful in some patients increasing the hemoglobin levels, as well as iron chelation to decrease the common iron overload observed in PKD patients [34]. However, in some severe cases, allogeneic bone marrow transplantation is required and it has been successfully performed in one severe affected child [12].

The feasibility of gene therapy in PKD was first reported by the group of Asano, who introduced the human LPK cDNA into C57BL/6 mouse bone marrow cells using a retroviral vector [7]. They demonstrated the expression of the LPK transgene mRNA in both peripheral blood and hematopoietic organs after bone marrow transplantation. However, viral-derived expression in peripheral blood was detectable no longer than 30 days post-transplantation, indicating an insufficient transduction efficacy of the retroviral vector used or transduction of non-pluripotent BM cells. In a hemolytic anemia dog model, bone marrow transplantation of minimal conditioned receptors failed to correct the hematological symptoms [10]. Other approaches to rescue RPK phenotype through a gene addition strategy have been also addressed using a PKD transgenic mouse model (*CBA/N PK-1^{SLC}/PK-1^{SLC}*) [9]. In this assay, the hemolytic anemia and reticulocytosis was fully corrected when the human gene was highly expressed by means of pronuclear injection, although splenomegaly was still present. Interestingly, the authors observed a negative correlation between RBC PK activity and the number of apoptotic erythroid progenitors in the spleen, providing evidence that the meta-

bolic alteration in PK deficiency affects not only the survival of RBC, but also the maturation of erythroid progenitors, resulting in ineffective erythropoiesis [35]. Further studies from this group indicate that RPK plays an important role as an antioxidant during erythrocyte differentiation, since glycolytic inhibition by mutations in *Pklr* gene increased the oxidative stress in SLC3 cells (established from *Pk-1*^{slc} mouse) and led to the activation of hypoxia-inducible factor-1 (HIF1), as well as the expression of downstream proapoptotic genes [36].

In addition, our work carried out in mouse models supported the therapeutic potential of viral vectors for the gene therapy of PK deficiency. Throughout the transduction of bone marrow cells using γ-retroviral vectors that carry the human RPK cDNA and subsequent transplantation, we reported a long-term expression of the human protein in RBC obtained from primary and secondary receptor mice, without detectable adverse effects [11]. Recently, we have also reported a successful gene therapy approach using the same retroviral vectors in the congenital mouse strain AcB55, identified by Min-Oo in studies of alleles involved in malaria susceptibility [37]. These mice carry a loss-of-function mutation (269T-> A) resulting in the amino acid substitution I90N in the *Pklr* gene, which yields a similar RBC phenotype to that observed in PKD patients, including splenomegaly and constitutive reticulocytosis. Retroviral-derived expression was capable of fully resolving the pathological phenotype in terms of hematological parameters, anemia, reticulocytosis and splenomegaly, together with normalization of bone marrow and spleen erythroid progenitors, erythropoietin (EPO), PK activity and ATP levels. Interestingly, despite a strong viral promoter was used to drive the expression of the transgene, metabolic energy balance was no modified in white blood cells. Moreover, we observed that values above 25% of genetically corrected cells were needed to fully rescue the deficiency [3], suggesting that RPK transfer protocols will always require a significant extent of gene-complemented HSC. Nevertheless, other experiments performed in the *CBA/N PK-1*^{SLC}/*PK-1*^{SLC} mouse model of PKD have reveled that 10% of normal BM renders RBC expressing nearly normal RPK protein levels [5]. Differences in the genetic defect of the mouse models used could account for these discrepancies, reinforcing the need for high transduction efficiencies to address the disease in the heterogeneous human population. Additionally, we have proposed the *in utero* transplantation of gene corrected cells as an alternative option for the treatment of PKD. The transplantation of RPK deficient lineage negative fetal liver cells transduced with lentiviruses (LVs) expressing the human wild type version of the RPK in 14.5 day-old fetuses partially restored the anemic phenotype, mainly due to a low engraftment of corrected cells [13]. Improved *in utero* cell transfer would allow therapeutic levels, thus offering an alternative therapeutic option for prenatally diagnosed severe PKD. Following our results in the AcB55 mouse model of PKD, phenotype correction could be reached if the percentage of engraftment of corrected cells is significant. We are currently developing improved lentiviral vectors that could be applied in future clinical settings.

Glucose phosphate isomerase (GPI) deficiency is the third most common hereditary cause of CNSHA, due to mutations in *GPI* gene located on the long arm of chromosome 19. The prevalence of this disease is still unknown, with no more than 50 cases reported so far, and with a higher incidence in the black population. The enzyme catalyzes the reversible isomer-

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ization from glucose 6-phosphate to fructose 6-phosphate, an equilibrium reaction of the glycolysis pathway. Glucose turnover is affected only in deficiencies below a very low critical residual GPI activity, but with a drastic decline of lactate formation. As no isoenzyme does exist, patients suffer not only from CNSHA and tissue hypoxia, but also from neuromuscular disturbances. In some cases, GPI deficiency has been found in PKD patients, increasing the severity of the clinical scenario and reflecting the degree of the perturbation of glycolysis. The lack of ATP leads to a destabilization of the erythrocyte membrane causing earlier lysis of the RBC and hemolytic anemia of variable degrees [38]. Animal models of GPI deficiency have been described, showing similar symptoms to the human disease [39]. Until now, no gene therapy attempt has been applied to this deficiency.

Other enzyme deficiencies causing CNSHA are Triose phosphate isomerase (TPI) deficiency, associated with neuromuscular disorders, mental retardation and frequent infections, Hexokinase deficiency (HK), affecting also platelet metabolism, phosphofructokinase (PFK) deficiency, 2,3-bisphosphoglycerate mutase (BPGM) deficiency and Glutathione synthetase (GS) deficiency (reviewed in [17,40,41]). Although the incidence of these diseases can be high (ie. TPI is considered as a frequent enzymopathy affecting 0.1% for caucasian populations and even 4.6% for black populations), they are considered rare or very rare diseases, because only few cases (~25 patients in the case of TPI) are diagnosed due to the severity of the clinical manifestations. No gene therapy approaches have been addressed up to now to treat these enzymopaties. However, due to their common characteristics, strategies developed in the other enzyme deficiencies could be applied directly to the treatment of all of them.

3. Optimization of vectors for the gene therapy of metabolic erythroid diseases: Erythroid specific expression vectors

The introduction of a cDNA, encoding for the correct version of the target mutated gene into patient cells using retroviral vectors has been successful for several inherited diseases. The initial integrative vectors for gene therapy design and used in clinical trials were based on Gamma(γ)-retroviral vectors in which the transgene expression was driven by the viral LTR promoter. γ-retroviruses preferably integrate in regions adjacent to the transcription initiation site [42]. The expression of the transgene is promoted by the viral LTR, which drives a high expression that can affect gene regulation of the surrounding genes. Although a high efficiency of transduction and therapeutic effects have been described with these vectors in various monogenic disorders such as immunodeficiencies, adverse effects associated with insertional mutagenesis have also been observed. This has led to the development of the next generation of integrative vectors using self-inactivating-LTR lentiviral backbones. SIN-Lentiviral vectors tend to integrate in intergenic transcribing areas, which represent a safer integrative pattern than γ-retroviral vectors. Aditionally, the expression of the transgene is driven by internal promoters, offering a more physiological expression and a less genotoxic profile when using weak promoters [43]. Current efforts to reduce the mutagenic potential of gene therapy vectors are focussed on not only the use of new viral backbones [44] but also on tissue-specific promoters to restrict the transgene expression to target cells [45] and insu-

lators to confer position-independent expression [46]. Additional regulatory DNA elements such as locus control regions (LCR), enhancers, or silencers have also been used to increase lineage specificity.

Gene therapy for RBC disorders requires, ideally, high erythroid-specific transgene expression in order to avoid side effects in progenitors or hematopoietic lineages other than the erythroid one. In inherited enzymophaties, the overexpression of metabolic enzymes in non-erythroid cells could provide these cells with a potential energetic advantage, with the consequent risk of disturbing the physiological generation of ATP in WBC. Also, the restriction of transcriptional activity to target cells with the use of either tissue-specific or physiologically regulated vectors decreasees the effect of the integrative vectors in the host genome. This goal is particularly important for erythrocyte metabolic deficiencies, as all the affected enzymes are highly regulated and connected with central metabolic pathways. Indeed, an expression limited to the erythroid progeny would reduce the genotoxic risk, as RBC become transcriptionally inactive during differentiation, and finally extrude their nucleus. To study tissue-specific gene therapy strategies for RBC diseases, hemoglobinophaties have been the most widely used.

Erythroid regulatory elements have been extensively used to manage targeted expression to RBC using reporter genes (Table 2). The Locus Control Regions (LCR), defined by their ability to enhance the expression of linked genes to physiological levels in a tissue-specific and copy number-dependent manner at ectopic chromatin sites are commonly used. The components of the LCR normally colocalize to sites of DNase I hypersensitivity (HS) in the chromatin of expressing cells. Individual HS are composed of arrays of multiple ubiquitous and lineage-specific transcription factor-binding sites. In early experiments performed with retroviral backbones, the group of Ferrari developed an erythroid-specific vector by the replacement of the constitutive retroviral enhancer in the U3 region of the 3′ LTR with the HS2 autoregulatory enhancer of the erythroid GATA-1 transcription factor gene. The expression of this vector was restricted to the erythroblastic progeny of both human progenitors and mouse-repopulating stem cells [47,48]. Later, they showed that the addition of the HS1 enhancer to HS2, both from the GATA-1 gene, within the LTR of the retroviral vector significantly improved the expression of the reporter gene. Another enhancer element that has been used to achieve erythroid-specific expression is HS40, located upstream of the ζ-globin gene, since it is able to enhance the activity of heterologous promoters in a tissue-specific manner [49]. It has been shown to be genetically stable in MMLV vectors and enhances expression comparable to that of a single -globin gene [50], although HS40 lacks some of the properties of the LCR, like position independence [51] or copy number dependence [52].

An additional improvement to provide safer vectors for RBC gene therapy was provided by the use of insulators elements, which have been shown to reduce position effects in transgenic animals [60]. Insulators are genomic elements that can shelter genes from their surrounding chromosomal environment, by either blocking the action of a distal enhancer on a promoter [60,61], or by acting as barriers that protect the gene from the silencing effect of heterochromatin [61]. The most well studied element is the chicken hypersensitive site 4

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(cHS4), an insulator sequence of the chicken -like globin cluster. Studies performed by Chung et al with the γ-globin promoter and the neo reporter gene on selected cells lines, demonstrated the ability of cHS4 to insulate the expression cassette from the effects of a strong -globin LCR element [63] and therefore reducing its genotoxicity. Experiments from Arumugam et al showed a two-fold reduction in transforming activity with insulated LCR-containing lentiviral vectors comparing with vectors lacking the cHS4 element [68].

Erythroid tissue-specific vectors			
Promoter / enhancer	**transgene**	**Vector type**	**Reference**
HS2 GATA-1 enhancer within the LTR	ΔLNGFR and Neo^R / EGFP	SFCM retroviral vector	[47]
HS1 to HS2 GATA-1 enhancer within the LTR	EGFP and hΔLNGFR	SFCM retroviral vector	[48]
Ankyrin-1 and α-spectrin promoters combined or not with HS40, GATA-1, ARE and intron 8 enhancers	EGFP	HIV-1 based vectors	[53]
α-globin HS40 enhancer and Ankyrin-1 promoter	GFP / *FECH*cDNA	HIV-1 based vectors	[4]
IHK, IHβp and HS3βp chimeric enhancers/ promoters	hβ-globin cDNA	Sleeping beauty transposon	[54]
Physiologically regulated vectors			
Promoter / enhancer	**transgene**	**Vector type**	**Reference**
HSFE and β-globin promoter	hβ-globin cDNA	MSCV retroviral vector	[55]
LCR and β-globin promoter	hβ-globin cDNA or EGFP	HIV-1 based vectors	[56,57]
β-globin and θ-globin promoters combined or not with HS40, GATA-1, ARE and intron 8 enhancers	EGFP	HIV-1 based vectors	[53]
LCR HS4, HS3, HS2, β-globin promoter and truncated β-globin intron 2	EGFP	HIV-1 based vectors	[58]
LCR, cHS4 and β-globin promoter	hβ-globin cDNA	HIV-1 based vectors	[46]
β-globin promoter, LCR HS2, HS3, HS4	hβ-globin cDNA	AAV2	[59]

LTR, long terminal repeats; HS: hypersensitive site; IHK, human *ALAS*2 intron 8 enhancer, HS40 from αLCR and ankyrin-1 promoter; Ihβp, human *ALAS*2 intron 8 enhancer, HS40 from αLCR and β-globin promoter; HS3βp, HS3 core element form human βLCR and β-globin promoter; LCR, locus control region. Modified from Toscano *et al.*, 2011

Table 2. Specific vectors for gene therapy of erythroid inherited diseases.

Tissue-specific expression using alternative human promoters can be convenient or more efficient for some diseases, but driving the expression of the therapeutic genes using own promoters is still the most physiological approach to reduce the genotoxic risk of integrating gene vectors [62]. The use of physiologically regulated vectors has been limited mainly because the promoter and the enhancer elements have to be obtained from the affected genes and they are often too large to be included in a lentiviral backbone, and also because the gene expression pattern depends partially on chromatin positioning [63]. -globin LCR has been widely used when attempting to solve this limitation. The -globin LCR consists of 5 HS regions located upstream of the entire cluster of human -like globin genes, each containing a high density of erythroid-specific and ubiquitous transcription binding elements [64]. Much of the transcriptional activity of the -globin LCR resides in HS2 and HS3 sites, but site 4 is important in adult globin expression [65]. Previous studies *in vitro* and *in vivo* have shown that -globin LCR can enhance erythroid-specific expression from heterologous non-erythroid promoters [66,67]. First approaches using -globin LCR and 3' enhancers were based on murine γretroviral vectors [74,75], but the limited packaging capacity of these vectors (up to 8 kb) did not allow the presence of such as large regulatory sequences. Several vector designs including different combinations of regulatory sequences and a deletion of a cryptic polyadenylation site within intron 2 of -globin gene [68], flanked by an extended promoter sequence and the -globin 3' proximal enhancer were developed. The combination of the LCR elements (3'2 kb) spanning HS2, 3 and 4, were the best amongst several possibilities [69] to achieve a high titer retroviral vector capable of expressing high levels of the transgene.

Other approaches to achieve consistent long-term expression of a transgene have been based on the use of HSFE element, an erythroid-specific chromatin remodelling element derived from the human β-globin LCR which contains binding sites for the erythroid-specific factors NF-E2, GATA-1, EKLF and the ubiquitous factor Sp-1, all of which are necessary to establish a hypersensitive chromatin domain. Work by Nemeth *et al.*, demonstrated that the HSFE can mediate functional tissue-specific "opening" of a minimal human β-globin promoter and increases expression of a human β-globin gene in both MEL cell clones and in transgenic mice. Their results indicated that the most effective vector included tandem copies of the HSFE and produced a 5-fold increase in expression compared to the promoter alone [55] in the context of an integrated retroviral vector.

Gene therapy for RBC metabolic diseases can also benefit from the new technologies based on the modification in mRNA stability or translation efficacy of the transgenes. The use of the post-transcriptional regulatory element (Wpre) from the woodchuck hepatitis virus (WHV) has significantly increased transgene expression in target cells [64,65], even in HSC [70] by stabilization of mRNA at post-transcriptional level. However, it may raise safety concerns, since it contains a truncated form of the WHV *X* gene, which has been implicated in animal liver cancer [71]. Therefore, Wpre has subsequently been improved by a mutation of the open reading frame of the *X* gene [72]. Combination of erythroid promoters like ankyrin-1 or -spectrin with Wpre sequence increased 2-fold the expression in unilineage erythroid cultures [53], and when combined also with erythroid enhancers inserted in tandem: HS40 and GATA-1 or HS40 and I8 enhancers [53]. RNA targeting strategies have mainly been used to down regulate expression of cellular genes using vectors expressing interference RNAs (iRNAs). They can be also used to control the expression of integrating vectors knocking down the transgene by the

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endogenous microRNA cellular machinery. Following this strategy, engineered microRNA target sequences in the vector (miRTs-vector) are recognized by a cell specific microRNA (miRNA), avoiding the expression of the therapeutic gene in undesired cell populations [63]. Several miRNAs are differentially expressed during hematopoiesis and their specific expression regulates key functional proteins involved in hematopoietic lineage differentiation. Particularly, miR-223 has been proposed as a myeloid-specific regulator that negatively regulates progenitor proliferation and granulocyte differentiation and activation [73]. Moreover, Felli et al observed that hematopoietic progenitor cells transduced with miR-223 showed a significant reduction of their erythroid clonogenic capacity, suggesting that down-modulation of this miRNA is required for erythroid progenitor recruitment and commitment [79]. Further studies may determine if the use of miRNA-223 target in lentiviral vectors could be useful to achieve a desirable erythroid-specific expression for gene therapy of red blood cell diseases.

In addition, the erythroid-specificity of short segments of the -globin LCR element has been documented in adeno-associated virus 2 (AAV2) system. Their efficacy to mediate an erythroid-restricted expression has been proved by Tan *et al.*, who reported a successful AAV2-mediated high and stable transduction of the human -globin gene in HSCs from -thalassemia mouse model, which were then transplanted into recipient and rescued them of the disease [59]. These vectors have gained attention as potential useful vectors for human gene therapy, mainly because of their non-pathogenic nature in humans and their relativly easy production. Besides, AAV2 vectors are easily purified to high titers and are able to transduce dividing and non-dividing cells. However, most of proviral AAV2 genomes remain episomal and the insert size is restricted to just over 4kb. Further studies are still needed to know whether they would be a better option than current lentiviral vectors. Also, long-term genotoxic risk of recombinant AAV2 therapy in human is not known up to the date.

In addition, the efficacy of some of these erythroid-specific elements and promoters has also been tested in non-viral vectors, such as transposons. Zhu et al, for instance, studied several hybrid promoters driving the expression of the human -globin gene using the sleeping beauty transposon (SB-Tn). They combined several erythroid elements to develop different chimeric promoters. Their results indicated that the ankyrin-1 minimum promoter was stronger than -globin's, and the hALAS I8 enhancer (IH) was significantly more powerful that HS3 core element from -LCR and -globin promoter [54]. SB-Tn system is a promising non-viral vector for efficient genomic insertion, even with erythroid-specificity. However, its efficiency for delivering transgenes into HSCs is still much lower than other engineering viral vectors.

4. Overcoming conventional gene therapy pitfalls: gene editing in induced plutipotent stem cells

4.1. Human induced pluripotent stem cells and reprogramming platforms

Since Yamanaka et al first reported the generation of mouse induced Pluripotent Stem Cells (iPSC) in 2006 by the ectopic expression of four transcription factors (Oct4, Sox2, Klf4 and

cMyc) [74] and one year later in human cells together with Thompson's group [75,76], many laboratories around the world have been able to reprogram a large range of somatic cells into pluripotent stem cells, from neural stem cells [77] to terminally differentiated B-lymphocytes [78]. The reproducibility and potentiallity (unlimited self-renewal and ability to differentiate into any cell type) of this technology has made the iPSC field to advance very rapidly. The human iPSC (hiPSC) technology brings together all the potential of hESC in terms of pluripotency without any ethical issue and the immunotolerance of the autologous cell treatment. Therefore, hiPSC technology is one of the most promising fields for future therapies for many human diseases. Safer reprogramming approaches have been designed and many patient specific hiPSC have been generated both to model human diseases and to correct by gene therapy approaches. Depending on the cell type to be reprogrammed, the number of factors used could be reduced and, what is more important, oncogenes or tumor related proteins included in the reprogramming cocktail, like *c-MYC* or *KLF4* [79] could be removed from the original reprogramming cocktail [80-82]. Several groups developed excisable polycistronic lentiviral vectors [83,84] or transposon-based reprogramming systems [85,86], which could be removed after getting the hiPSC clones. Similar results have been obtained using recombinant proteins [82], synthetic mRNAs [87], and non integrating RNA Sendai Virus vectors [88]. Except for Sendai viruses, non integrating methods show a reduced reprogramming efficiency and the range of cells reprogrammed is not as large as with lentiviral or retroviral vectors.

iPSC technology makes feasible the availability of patient specific cells to study the biology of the disease and develop advanced tools to cure the phenotype and could potentially be used as a therapeutic option (Figure 1). Focussing on metabolic diseases, the first reported metabolic disease patient specific hiPSC line was obtained one year after the first generation of hiPSCs. It was from a 42-year old female that suffered from Type I Diabetes mellitus [89] and it showed no differences compared to a wild type hiPSC line in terms of pluripotency. Next report in which liver metabolic disease patient samples were reprogrammed was carried out by the group of Ludovic Vallier [90], and showed the potential of this kind of approaches for disease modelling and new drug discovery. They reprogrammed fibroblast obtained from α-1 Antitrypsin deficiency (*A1ATD*), Familiar Hypercholesterolemia (FH), Glucose-6-Phosphate deficiency (G6PD), Crigler-Najjar Syndrome and hereditary Tyrosinemia Type 1 patients, and generated hepatocytes that showed characteristics of mature hepatic cells, like albumin secretion or cytocrome p450 metabolism. Three of the five cell lines (*A1ATD*, FH, and GSD1a hiPSCs) were capable of recapitulating the disease phenotype in vitro. Disease modelling in erythroid diseased induced pluripotent cell lines has been performed for -Thalassemia [91,92] and sickle cell anemia [93,94]. In these reports the phenotype was corrected by LVs integrated in areas of the genome that were considered safe for viral integration [83] or by gene editing using homologous recombination of the affected locus [91,93,94].

The future therapeutic application of hiPSC will not only require non-integrative reprogramming system, but also a more precise gene correction. During last years, the cooperation between hiPSC technology and gene editing is being explored. Human iPSC technology has

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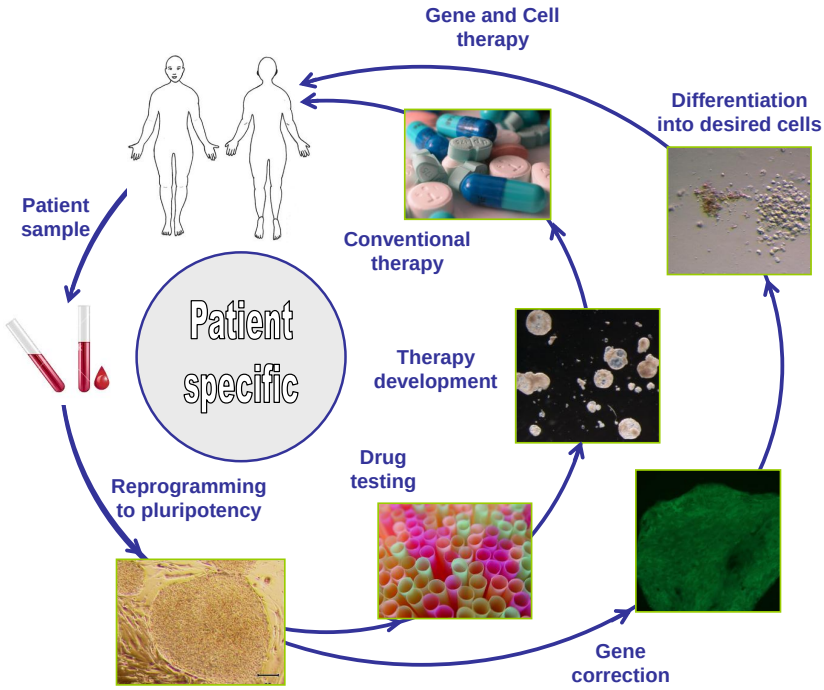

Figure 1. Potential utilities of hiPSC and iPS technology

led to the opportunity to control the integration of viral vectors at a clonal level. As we have mentioned before, the analysis of lentiviral integration sites in β-thalassemia hiPSC allowed the identification of corrected hiPSC clones expressing β-globin transgene from a safe genomic site (also called Safe harbour), a site in which integration does not disturb the expression of any neighbouring genes during their erythroid differentiation [83]. The therapeutic use of patient-specific hiPSC emerges then from the combination of gene and cell therapy. From this new research field,future gene therapy protocols will emerge.

4.2. Gene editing based on homologous recombination

Gene editing is a process in which a DNA sequence is introduced into a specific locus or a chromosomal sequence is replaced. This site-specific precise introduction requires an accurate recognition mechanism of the target site on the genome. Under normal conditions, the maintenance of the integrity of the genome requires that the cells repair DNA damage with high fidelity. One of the most harmful DNA damage is the generation of double-strand breaks (DSB). DSB are often resolved by non-homologous end joining (NHEJ), which joins the two ends of the DSB. However this DNA repair mechanism could introduce mutations. On the contrary, homologous recombination (HR) is a truly accurate DNA repair mechanism because it is basically a “copy and paste” mechanism. This process uses an undamaged

homologous segment of DNA that can be exogenously provided as a template to copy the information across the DSB. The fidelity of HR gives us the specificity and accuracy that gene editing requires.

The natural HR process has been adapted by researchers to get the desirable addition of an exogenous cassette into the targeted locus. This techniques have been widely use for the generation of knock-out and knock-in transgenic animals [95]. To correct or insert and express a transgene by HR we can consider three different strategies: i) Gene correction, a base or some bases can be substituted from the original strand using an homologous sequence where this base or bases are modified; it is the way to introduce/repair point or small mutations; ii) Safe harbour integration, a complete expression cassette (promoter, transgene and regulatory signals) is inserted in a safe place of the genome, without altering the expression of the surrounding genes and without being silenced by epigenetic mechanisms; this is the case for *AASV1* and *CCR5* loci. Additionally to these well known safe harbours, there is a wide research focused on finding potential new safe harbour places. iii) Knock-in insertion, the cDNA of a gene is introduced in the same site of the endogenous gene, linked by splicing mechanisms to the endogenous gene assuring the expression of the inserted sequence by the endogenous regulatory elements of the locus where it is integrated.

Gene editing process can be separated in two different steps, generation of DSB and HR. The efficacy of gene editing in human cells depends on the generation of DSB at the specific target site and on the DNA repair mechanism that the cell uses to resolve the DSB. Unfortunately, NHEJ is the dominant pathway to solve these DNA lesions in human cells. Additionally, HR varies in different cell types and requires transit through S-G2 phase of the cell cycle [96]. These limitations make gene editing in human cells difficult to achieve. However, different approaches are being used to improve gene editing by HR, like increasing the length of the DNA sequences homologous to target site (homology arms) [97], the use of adeno-associated vectors [98], the improvement of selection methods for edited cells or the stimulation of HR by inducing DSB using DNA nucleases.

Recently, engineered DNA nucleases have been developed to specifically induce DSB at a unique and defined sequence in the cell genome. These proteins are formed by a nuclease domain and a DNA binding domain whose sequence specificity can be engineered. The most widely used DNA nucleases are Zinc finger nucleases (ZFN), homing meganucleases (MN) and transcription activator-like effector nucleases (TALEN). They identify a potentially unique sequence in the genome and generate DSBs in the desired genomic site, aiming to promote the repair of the DSB by the cell machinery and, ideally by HR. The DNA binding domain of a ZFNs is derived from zinc-finger proteins and is linked to the nuclease domain of the restriction enzyme Fok-I. DNA-biding domain is a tandem repeat of Cys_2His_2 zinc fingers, each of which recognizes three nucleotides. ZFNs work as pairs of two monomers of ZFN, one in reverse orientation. This ZFN dimer can be designed to bind to genomic sequences of 18-36 nucleotides long. TALENs have a similar structure to ZFNs, but the DNA-binding domain comes from transcription activator-like effector proteins. The DNA-binding domain in TALENs is a tandem array of amino acid repeats. Each of these units is able to bind to one of the four possible nucleotides and this makes that the DNA binding domain can be designed to recognize any desired genomic sequence. TALENs also cleave as dimers. Contrary to these synthetic DNA-nucleases, MNs are a

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subset of homing endonucleases which recognize a DNA sequence from 14 to 40 nucleotides. Current MNs have been engineered from natural homing endonucleases to increase the number of target DNA sequences.

ZNFs have been widely used for gene editing in hESC and hiPSC. In 2007, Dr. Naldini's laboratory showed the insertion of GFP into the CCR5 safe harbour in human stem cells (HSC and hESC) after inducing HR by ZFN expression. The CCR5-ZFN and donor DNAs were delivered into hESC by intergrative deficient lentiviruses. More interestingly, targeted hESC were able to differentiate into neurons keeping GFP expression [99]. Soon, the proof of principle for the clinical application of ZFN-mediated gene editing was tested in hiPSC from patients affected by different genetic diseases. The first pre-clinical use of ZFN for gene therapy of a metabolic disease was performed by Yusa *et al*. In this report, gene correction was performed at the α1-antitrypsin (*A1AT*) locus to revert A1AT deficiency in hiPSC derived from a patient with a point mutation. This group included a Puromycin resistence cassette flanked by piggyBac sites, so that the Puromycin selection facilitated the isolation of corrected A1ATD-iPSC clones. Afterwards, the selection cassette was removed by piggyBac transposon, obtaining corrected hiPS clones without any additional sequence. These corrected hiPS clones were then differentiated into hepatocyte-like cells to confirm the complete correction of the A1ATD [101]. Other hiPSC gene editing approaches and functional correction of erithroid diseases include gene correction of Sickle Cell Anemia [94] and -Thalassemia [91].

One of the major limitations of ZFN is the generation of "off-target" DSB, due to unspecific sequence recognition. Different studies have highlighted this as a possible limitation in the clinical use of ZFN-mediated HR [100,101]. Recent works have explored the potential of other types of DNA-nucleases in order to prevent the "off-target" cleave limitations of the ZFN, being TALEN and MN the most promising ones. The feasibility of TALEN to mediate HR in hESC and hiPSC was assess by Jaenisch's group when they designed TALEN targeting the *PPP1R12C* (at *AAVS1* locus), *POU5F1* and *PITX3* genes at precisely the same positions as the one targeted by ZFN in their previous work [102]. The authors described a gene editing efficiency similar to the one achieved by ZFN with a low level of "off-targets" [103]. A strategy to minimize the potential number of "off-targets" is to design TALEN to work as obligatory heterodimers, which has beeing already done in the engineered MNs. The application of the TALEN and MN as tools to improve HR is still on going. We are exploring the pre-clinical use of TALEN and MN to correct erythroid metabolic genetic diseases, such as PKD.

5. Complementary developments for the application of gene therapy to erythroid metabolic diseases

5.1. *In vivo* transduction using engineered envelopes

Another challenge for the clinical application of gene therapy relates to vector targeting. To achieve successful gene therapy, the appropriate gene must be delivered to target cells and specifically expressed in them, without harming non-targeted cells. The most common and easiest way to target specific cells is by *ex vivo* infection of the desired cell population. There-

fore, cells can be directly exposed to the viral vectors facilitating viral-cell interaction. These interactions are driven by the envelope protein which can be adapted from other viruses redirecting the tropism of the vector. The most widely used vectors are lentiviral vectors pseudotyped with the attachment glycoprotein of the vesicular stomatitis virus (VSV-G), which allows the production of high-titre vectors and confers a broad host range [104]. In comparison with them, engineered LVs capable of delivering genes of interest to predetermined cells, can reduce the targeting of undesirable cell types and improve the safety profile, which will further enhance the use of this vector system for gene therapy applications [105,106]. As we have mentioned above, the use of promoters and regulatory sequences that are only active in target cells adds lineage specific expression, although integration of the viruses in non desired cells is still possible. *Ex vivo*-targeted gene delivery, as commonly used in HSCs transduction, is associated with a risk of inducing cell differentiation and loss of the engraftment potential of these cells [107]. On the contrary, *in vivo* gene transfer could target HSCs in their stem cell niche, a microenvironment that regulates HSC survival and maintenance [105]. To accomplish this, the vector must display a suitable system to selectively infect the desired population, for example the introduction of a specific ligand to bind a target-cell receptor [106].

Many attempts have been made to develop targeted transduction systems using retroviral and lentiviral vectors by altering the envelope glycoprotein (Env), which is responsible for the binding of the virus to the cell surface receptors and for mediating viral entry into the cell. The plasticity of the surface domain of Env allows insertion of ligands, peptides or single-chain antibodies that can direct the vectors to specific cell types [108]. However, this type of manipulation negatively affects the fusion domain of Env, resulting in low viral titers. To overcome this downside, a method to engineer lentiviral vectors has been developed. These vectors transduce specific cell types by breaking up the binding and fusion functions of the envelope protein into two distinct proteins [108]. Instead of pseudotyping lentiviral vectors with a modified viral envelope protein, these lentiviral vectors co-display a targeting antibody and a fusogenic molecule on the same viral vector surface. Based on molecular recognition, the targeting antibody should direct lentiviral vectors to the specific cell type. The binding between the antibody and the corresponding cellular antigen should induce endocytosis resulting in the transport of lentiviral vectors into the endosomal compartment. Once inside the endosome, the fusogenic molecule should undergo a conformational change in response to the decrease in pH, thereby releasing the viral core into the cytosol [109]. The use of fusion domain of the binding defective Sindbis virus glycoprotein together with an anti-CD20 antibody has been shown to mediate the targeted transduction of lentiviral vectors to CD20-expressing B cells [110].

However, two major challenges for *in vivo* gene delivery are LVthe exposure to the host immune/complement system and off-target cell transfer after systemic administration. For these reasons, second generation of early-acting-cytokine-displaying LVs has been developed, that circumvents these obstacles by specifically targeting $hCD34^+$ cells [111,112]. For example, RDTR/SCFHA-LV, consisting of RD114 glycoprotein and stem cell factor (SCF) fused to the *Influenza hameglutinin* env protein, is resistant to degradation by human comple-

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ment and efficiently transduces very immature hCD34$^+$ HSCs [113]. This new generation of HSC-targeted LVs should improve current gene therapy protocols through the transduction of primitive HSCs directly in the bone marrow of patients with genetic diseases.

5.2. *In vitro* production of mature erythrocytes

Periodical blood transfusion is the previous to the last therapeutic option for severe cases of CNSHA patients. However, this clinical practice involves also adverse effects related to the immuneresponse against minor erythrocyte antigens which makes the patients refractory to additional blood transfusions in the long run. The availability of genetically corrected patient-specific iPSC would allow the possibility of generating disease free erythrocytes ready for transfusion, avoiding the adverse immune effects.

There have been numerous attempts to produce RBC *in vitro* from different sources of stem cells. To date, the most successful protocol has been developed by the group of Luc Douay [113,114]. Using peripheral blood CD34$^+$ cells, these authors were able to expand and generate RBC with *in vitro* and *in vivo* features of native RBC, and were also capable of transfusing a patient with *in vitro* generated erythrocytes. Notably, the same authors reported a protocol to generate RBC from hiPSC as an alternative source of HSC [114]. Other groups have described similar protocols to generate erythrocytes from hESC or hiPSC [115-118], although in all these studies the RBC generated from embryonic cells expressed embryonic and foetal hemoglobins but low levels of adult hemoglobin. Additional efforts should be done to make this possibility a therapeutic option.

6. Conclusions

Erythroid metabolic diseases are well defined and well known diseases which main symptom is CNSHA. As they are monogenic diseases that can be cured by allogeneic bone marrow transplantation, they are very good candidates to be treated by gene therapy. However, the low number of patients with poor prognosis requiring BM transplantation and the absence of an apparent selective advantage of the corrected cells over the diseased ones have made their approach for gene therapy less attractive than other erythropaties. Up to now, no gene therapy clinical trial for erythroid metabolic diseseases has been accomplished. Gene therapy attempts in animal models have been applied to G6PD and PKD with successful results, emphasizing the usefulness of a gene therapy approach for these diseases. Although adverse effects due to ectopic expression of the metabolic enzyme have not been observed, an erythroid specific expression is preferred. Many developments have been made for the specific expression of globin genes that could be adapted to vectors developed for the discussed erythroid metabolic diseases. Similarly, any attempt directed to the improvement of HSC transduction, including the possibility of *in vivo* targeted gene therapy could be applied. On the other hand, the combination of cell reprogramming and gene editing opens a new world of possibilities that could be easily applied to these diseases. hESC and hiPSC are helping in the development of the next generation of gene therapy, which implies a precise

gene targeting. Gene editing by HR is the best and safest gene therapy procedure because avoids any perturbation in the targeted genome. Besides the combination of hiPSC and gene editing could be the future therapy for many genetic-based diseases. The hiPSC technology is the springboard for the development of more efficient HR protocols applicable to other types of stem cells such as hematopoietic stem cells. The combination of methods for obtaining big amounts of RBC from HSC or embryonic cells, along with the improvement of the different gene therapy approaches described in this chapter, opens up the possibility of the therapeutic application involving the infusion of RBC differentiated *in vitro* from genetically corrected patient specific stem cells.

Nomenclature

5-FU 5-fluorouracil

A1ATD-1 antitripsin deficiency

AAV Adeno-associated virus

BM Bone marrow

BPGM 2,3-bisphosphoglycerate mutase

CNSHA Chronic non spherocytic hemaolotyc anemia

DSB Double strand breaks

Env Viral envelope

FH familiar hypercholesterolemia

G6P Glucose-6-phosphate

G6PD Glucose-6-phopahate dehydrogenase

GPI Glucose phosphate isomerase

GS Glutathione synthetase

hESC human embryonic stem cell

hIF1 hypoxia-inducible factor-1

hiPSC Human induced pluripotent stem cell

HK Hexokinase

HR Homologous recombination

HS DNase I hypersensitive sites

HSC Hematopoietic stem cell

iPSC Induced pluripotent stem cell

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kb kilobases

LCR Locus control region

LTR Long terminal repeats

LV Lentivirus

MN homing meganuclease

NHEJ non-homologous end joining

PFK phosphofructokinase

RBC Erythrocytes

SIN-LV Self-inactivated lentiviral vector

TALEN transcription activator-like effector nuclease

TPI Triose phosphate isomerase

WT wild-type

ZFN zinc finger nuclease

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