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Recent Advances in Hematopoietic Stem Cell Gene Therapy

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1. Introduction

Hematopoietic stem cell transplantation (HSCT) has a half-century history. It is currently an indispensable treatment for not only incurable blood diseases such as aplastic anemia and severe hemolytic anemia, but also malignant hematological diseases such as leukemia and lymphoma. Although allergenic HSCT is also used to treat hereditary diseases, its indications are restricted because of critical complications including regimen-related toxicities involving conditioning, infection, and graft-versus-host disease.

Studies in recent decades have shown that HSCT can have a long-term effect in the treatment of hereditary diseases involving a responsible gene in hematogenous cells. Although the first successful gene therapy using lymphocytes or bone marrow cells for a patient with adenosine deaminase (ADA) deficiency inspired great hope in the future of gene therapy [1-3], subsequent gene therapy using HSCs for patients with X-linked severe combined immunodeficiency (SCID-X1) resulted in tumorigenesis [4]. In addition to the self-renewal and multilineage differentiation capacities of tissue stem cells, HSCs exhibit cell-cycle dormancy, which complicates their use in gene therapy.

However, as technological advances have increased the safety and efficiency of introducing genes into HSCs, gene therapy with HSCs is attracting attention again. In this chapter, advances in the technology of HSC gene therapy, e.g., vector design to avoid genotoxicity and increase transgenic efficiency by taking advantage of the special characteristics of HSCs, are reviewed. In addition, recent studies on HSC gene therapy for various hereditary diseases, such as thalassemia, Fanconi anemia, hemophilia, primary immunodeficiency, mucopolysaccharidosis, Gaucher disease, and X-linked adrenoleukodystrophy (X-ALD) are discussed.



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2. Characteristics of HSCs and gene therapy

The concept of the HSC was introduced by Till and McCulloch in 1961 [5]. Although a healthy adult produces approximately 1 trillion blood cells each day, they are considered to originate from a single HSC which can potentially be transplanted into a mouse [6, 7]. Generally stem cells are defined as cells capable of self-renewal and multilineage differentiation. In addition to these two characteristics, HSCs have the capability of cell-cycle dormancy, i.e. to enter a state of dormancy (G0 phase) in the cell cycle and can continue blood cell production over a lifetime while protecting themselves from various kinds of stress [8].

Fig. 1 shows HSC surface markers and the typical cytokines regulating HSCs. Stem cell factor (SCF) and thrombopoietin (TPO) are important direct cytokine regulators of HSCs. Although SCF promotes the proliferation and differentiation of hematopoietic progenitor cells, it is thought to not be essential for the initiation of hematopoiesis and HSC self-renewal [9]. TPO and its receptor, c-Mpl, are thought to play important roles in early hematopoiesis from HSCs. In contrast to the CD34⁺CD38⁻c-Mpl⁻ population, CD34⁺CD38⁻c-Mpl⁺ cells show significantly better HSC engraftment [10]. Mice lacking either TPO or c-Mpl have deficiencies in progenitor cells of multiple hematopoietic lineages [11]. TPO-mediated signal transduction for the self-renewal of HSCs is negatively regulated by the intracellular scaffold protein Lnk [12, 13]. A signal from angiopoietin-1 via Tie2 regulates HSC dormancy by promoting the adhesion of HSCs to osteoblasts in the bone marrow niche and maintains long-term repopulating activity [14]. Although cytokine-induced lipid raft clustering of the HSC membrane is essential for HSC reentry into the cell cycle, transforming growth factor- β (TGF- β) inhibits lipid raft clustering and induces p57Kip2 expression, leading to HSC dormancy [15, 16]. Recently, the hypoxic niche of HSCs has been demonstrated. It, along with the osteoblastic and vascular niches, are important for HSC dormancy [17-19]. They are targets in HSC gene therapy [20].



Figure 1. Hematopoietic stem cell (HSC) surface markers and typical cytokines that regulate HSCs. Stem cell factor (SCF) promotes the proliferation and differentiation of HSCs. Thrombopoietin (TPO) and its receptor, c-Mpl, play important roles in early hematopoiesis, especially self-renewal. Signals from angiotensin-1 via Tie2 and transforming growth factor - β via its receptors regulate HSC dormancy. (This figure is based on the illustration by BioLegend, Inc. San Diego, CA, U.S.A. http://www.biolegend.com/cell_markers)

While making a HSC with few opportunities for cell division into a transgenic target, it is important to design a safe and efficient vector for inserting a gene into the host chromosome. Furthermore, since a hematogenous cell also has many cells which exhibit its function in the specialization process to a mature effector cell, it is also important to select differentiation-specific or non-specific promoters or enhancers during the vector design process.

3. Vectors for HSC gene therapy

Vectors derived from the Retroviridae family, RNA viruses with reverse transcriptase activity, are widely used for inserting genes in host chromosomes. Although adeno-associated virus (AAV) vectors can also insert genes into host chromosomes, this process is inefficient and partial. Gammaretroviruses and lentiviruses are members of the Retroviridae family that are commonly used as vectors in HSC gene therapy. Generally, the former is called simply a retroviral vector and the latter is called a lentiviral vector. When a gene is inserted in the chromosome of an HSC with a Retroviridae vector, genotoxicity can occur.

3.1. Gammaretroviral (Retroviral) vectors

Retroviral vectors are commonly constructed from the Moloney murine leukemia virus (MoMLV) genome. Retroviral genomes have a *gag/pol* gene that codes for viral structure proteins, protease and reverse transcriptase, an *env* gene that codes for the envelope glycoprotein and the packaging signal. These genes are flanked by long terminal repeats (LTR) which contain enhancers and promoters. A retroviral vector consists of a packaging plasmid that does not have the packaging signal but does include the *gag/pol* gene, a transfer vector with the packaging signal, and the target gene cDNA. After transfection of these plasmids into producer cells (e.g., 297T cells, NIH3T3 cell, etc.), a target vector is obtained by collecting the culture solution.

Expression of a target gene can be inhibited by mechanisms such as methylation of CpG islands in the promoter region, insertion of a negative control region (NCR) into the LTR, and the presence of a repressor binding site (RBS) downstream of the 5' LTR. Other vectors, such as the murine stem cell virus (MSCV) vector [21], the myeloproliferative sarcoma virus vector, the negative control region deleted (MND) vector [22], and the MFG-S vector [23] were developed to improve the efficiency of transgene expression; they are widely used in clinical applications of gene therapy involving HSCs.

Since the retroviral viral genome cannot cross the nuclear membrane, it can be incorporated into a chromosome only during the phase of mitosis when the nuclear membrane has disassembled. Since many HSCs are thought to exist in a dormant phase, insertions into the HSC genome with a retroviral vector require a proliferation stimulus by cytokines. Although various combinations of cytokines to suppress the decrease in HSC self-renewal have been studied, stem cell factor (SCF), fms-related tyrosine kinase-3 (Flt-3) ligand, interleukin-3 (IL-3), TPO, among others, are commonly used [24, 25].

3.2. Lentiviral vectors

Human immunodeficiency virus type 1 (HIV-1), the representative lentivirus, differs from gammaretroviruses in that it can be incorporated during a non-mitotic phase. This is one advantage of lentiviral vectors in HSC gene therapy.

Both lentiviruses and gammaretroviruses have *gag*, *pol*, and *env* genes sandwiched between LTRs with promoter activity at both ends. In addition, lentiviruses have accessory genes (*vif*, *vpr*, *vpu*, *nef*) and regulatory genes (*tat*, *rev*). Double-stranded cDNA produced from the viral genome enters the cell, and a pre-integration complex is formed with a host protein. This complex can pass through the pores of the nuclear membrane during non-mitotic phases, allowing the viral genome to be inserted into the host cell chromosome.



Figure 2. HIV provirus (A) and the four plasmids of a third-generation lentiviral vector (B). The viral long terminal repeats (LTRs), reading frames of the viral genes, splice donor site (SD), splicing acceptor site (SA), packaging signal (Ψ), and rev-responsive element (RRE) are indicated. The packaging plasmid contains the *gag* and *pol* genes under the influence of the CMV promoter, intervening sequences, and the polyadenylation site (polyA) of the human β -globin gene. As the transcripts of the *gag* and *pol* genes contain cis-repressive sequences, they are expressed only if rev promotes their nuclear export by binding to the RRE. All *tat* and *rev* exons have been deleted, and the viral sequences upstream of the *gag* gene have been replaced. The rev plasmid expresses *rev* cDNA. The SIN vector plasmid contains HIV-1 cis-acting sequences and an expression cassette for the transgene. It is the only portion transferred to the target cells and does not contain wild-type copies of the HIV LTR. The 5' LTR is chimeric, with the RSV enhancer and promoter replacing the U3 region to rescue transcriptional dependence on *tat*. The 3' LTR has an almost completely deleted U3 region, which includes the TATA box. As the latter is the template used to generate both copies of the LTR in the integrated provirus, transduction of this vector results in transcriptional inactivation of both LTRs; thus, it is a self-inactivating (SIN) vector. The envelope plasmid encodes a heterologous envelope to pseudotype the vector, here shown coding for vesicular stomatitis virus (VSV)-G. Only the relevant parts of the constructs are shown (Reproduced with modifications from [26]). Although first-generation lentiviral vectors included modification genes, they were removed in the second generation because it was discovered that the modification genes are not required for infection during non-mitotic phases. In the third generation, further modifications included the deletion of *tat*, use of multiple vector plasmids, and introduction of selfinactivating (SIN) vectors. The structure of HIV-1 and a typical third-generation lentiviral vector system are shown in Fig. 2 [26]. Approximately one-third of the HIV-1 genome has been deleted, and the vector system has been divided into four plasmids, namely, the packaging plasmid, rev plasmid, SIN vector plasmid and envelope plasmid. To prevent production of wild type HIV-1, tat, a regulatory gene indispensable to viral reproduction was deleted, and the rev gene was moved to a separate plasmid. Moreover, since the HIV-1 LTR promoter is weak in the absence of *tat*, it was replaced with the cytomegalovirus (CMV) promoter in the packaging plasmid. Since an envelope plasmid can only infect CD4 positive cells with a HIV-1 envelope, the envelope gene was replaced with the vesicular stomatitis virus G glycoprotein (VSV-G) envelope. The SIN vector further improved safety by replacing the enhancer / promoter portion of the LTR, suppressing the activation of unnecessary genes with the integrated gene (Fig. 3) [27].



Figure 3. Mechanism of gene activation induced by vector insertion. The genomic integration site of an MLVbased retroviral vector is depicted. With this MLV vector design, the enhancer and promoter within the U3 region (blue rectangle) of the long terminal repeat (LTR) drive transcription of the transgene (indicated by the parallel arrow arising from the blue rectangle). Vector integration near Gene X is shown in the top panel. The enhancer elements located in the U3 region (blue rectangle) of the vector can interact with the regulatory elements upstream of Gene X to increase its basal transcription rate to inappropriately high levels, potentially altering the growth of the cell. Two alternatives for eliminating the use of the powerful enhancer in the U3 region include (1) middle panel: use of a selfinactivating (SIN) MLV-based vector in which the U3 region has been deleted. An internal cellular promoter is used to drive transgene expression and (2) bottom panel: use of a SIN lentiviral vector in which U3 (yellow rectangle) has been eliminated. This system also uses an internal cellular promoter to drive transgene expression (Reproduced with modification from [27]).

To improve the gene transfer into HSCs, Verhoeyen and colleagues designed lentiviral vectors displaying "early-acting cytokines" such as TPO and SCF. This vector can promote survival of CD34 positive HSCs and achieve selective transduction of long-term repopulating cells in a humanized mouse model (Fig. 4) [28, 29].



Figure 4. Lentiviral vector particles (HIV-1) display recombinant membrane envelope proteins such as stem cell factor (SCF), thrombopoietin (TPO), and vesicular stomatitis virus G glycoprotein (VSV-G). This vector can specifically target vector particles to hematopoietic stem cells (HSCs) expressing c-kit and c-mpl receptors for SCF and TPO, respectively. VSV-G envelope protein can bind to phospholipids in the HSC cell membrane. (Karlsson S, Gene therapy: efficient targeting of hematopoietic stem cells. Blood. 2005;106(10):3333)

3.3. Genotoxicity of viral vectors

The most serious problem with using viral vectors to incorporate a gene into a chromosome is the potential development of clonal proliferative diseases such as leukemia, which was observed in clinical trials involving gene therapy for SCID-X1 and chronic granulomatous disease (CGD). Although this problem of genotoxicity represents a great hurdle in the development of clinical applications for gene therapy, there is promising ongoing research on the mechanisms underlying genotoxicity and how to avoid it.

The mechanisms of retrovirus-induced oncogenesis are shown in Fig. 5 [30]. In oncogene capture, an acute transforming replication-competent retrovirus captures a cellular proto-oncogene and mediates transformation. This mechanism does not occur in replication-in-competent vectors. Second, the provirus 3' LTR can trigger increased transcription of a cellular proto-oncogene. Third, enhancers in the provirus LTRs can activate transcription from nearby cellular proto-oncogene promoters. Fourth, a novel isoform can be expressed

when transcription from the provirus 5' LTR creates a novel truncated isoform of a cellular proto-oncogene via splicing. Fifth, an inserted provirus can disrupt transcription by causing premature polyadenylation. The same mechanisms can occur in cellular oncogenesis when a gene is inserted by a retroviral vector [30].



Figure 5. Retroviral mechanisms of oncogenesis. The detailed mechanisms are shown in the text. The integrated provirus is indicated by two LTRs. Cellular proto-oncogene promoter and exons are indicated by black and grey boxes respectively (Reproduced from [30]).

Even if a gene is inserted into a HSC similarly, it is also known that there are diseases which may develop a tumor, and diseases a tumor is not accepted to be. Each type of virus has a unique integration profile, and the following observations have been made [30]: (a) Different retroviral vectors have distinct integration profiles. (b) The route of entry does not appear to strongly affect distribution of integration sites. VSV-G–pseudotyped HIV vectors have an integration profile similar to HIV virions with the native HIV envelope despite differences in the route of entry. (c) The integration profile is largely independent of the target cell type,

although the transcriptional program and epigenetic status of the target cell can influence integration site selection. (d) For lentiviruses, which can integrate independently of mitosis, the cell-cycle status of the target cell has only a modest effect on the distribution of integration sites.

In order to avoid genotoxicity, various SIN vectors have been developed and improved. In general, lentiviral vectors are considered to have a lower risk of oncogenesis than retroviral vectors [31]. However, when a HSC is the target cell, more attention should be required because tumorigenesis can occur when the cell with the inserted gene undergoes differentiation.

4. Clinical applications of HSC gene therapy

Diseases in which gene therapy using HSCs are being studied are shown in Table 1. They are roughly divided into hematological disorders, immunodeficiencies, and metabolic diseases. Most are congenital or hereditary diseases. The characteristic clinical features and recent basic science or clinical studies on HSC gene therapy for each disease are discussed below.

Congenital hematopoietic disorders
β-thalassaemia
Fanconi anemia
Hemophilia
Primary immunodeficiencies
X-linked severe combined immunodeficiency (SCID-X1)
Adenosine deaminase deficiency (ADA-SCID)
Chronic granulomatous disease (CGD)
Wiskott-Aldrich syndrome (WAS)
Janus kinase 3 (JAK3) deficiency
Purine nucleoside phosphorylase (PNP) deficiency
Leukocyte adhesion deficiency type 1 (LAD-1)
Congenital metabolic diseases
Mucopolysaccharidosis (MPS) types I, II, III, VII
Gaucher disease
X-linked adrenoleukodystrophy (X-ALD)

 Table 1. Clinical applications of hematopoietic stem cell gene therapy.

4.1. β-thalassemia

Hemoglobin A (HbA), comprising 98% of adult human hemoglobin, is a tetramer with two α -globin and two β -globin chains combined with a heme group. β -thalassemia is an

autosomal hemoglobin disorder caused by decreased β -globin chain synthesis. Although individuals with β -thalassemia minor (heterozygote) may be asymptomatic or have mild to moderate microcytic anemia, β -thalassemia major (homozygote) progresses to serious anemia by one or two years of age, and hemosiderosis, iron overload caused by transfusion or increased iron absorption, develops. Since most patients develop life-threatening complications such as heart failure by adolescence, HSCT has been performed in patients with advanced disease [32]. In recent years, gene therapy using a lentiviral vector containing a functional β -globin gene has been performed in an HbE/ β -thalassemia (β^{E} / β^{0}) transfusion-dependent adult male, who subsequently did not require transfusions for over 21 months [33].

The human β -globin locus is located in a large 70kb area which also contains some β -like globulin genes (ϵ , G γ , A γ , δ , β). Gene switching takes place according to the development stage, and the β -globin gene is transcribed and expressed specifically after birth. A powerful enhancer called the LCR (locus control region) exists on the 5' side of the promoter. The LCR contains five DNase I hypersensitive sites, referred to as HS5 to HS1 starting from the 5' side. Furthermore, HS5 contains CCCTC-binding factor (CTCF)-dependent insulator.

The structure of the lentiviral SIN vector used in gene therapy for β -thalassemia is shown in Fig. 6. To improve safety, two stop codons were inserted into the packaging signal (ψ) of GAG, the HS5 portion with insulator activity was deleted, and two copies of the 250 base pair (bp) core of the cHS4 chromatin insulators (chicken β -globin insulators) were inserted in the U3 region of the HIV 3' LTR. Furthermore, the amino acid at the 87th position of β -globin was changed from threonine to glutamine. This altered β -globin can be distinguished from normal adult β -globin by high performance liquid chromatography (HPLC) analysis in individuals receiving red blood cell transfusion and β ⁺-thalassemia patients [33].



Figure 6. Diagram of the human β-globin gene in a lentiviral vector. HIV LTR, human immunodeficiency type-1 virus long terminal repeat; Ψ +, packaging signal; cPPT/flap, central polypurine tract/DNA flap; RRE, rev-responsive element; β p, human β -globin promoter; ppt, polypurine tract; HS, DNase I Hypersensitive Sites (Reproduced with color modification from [33])

A clinical study using this vector was performed in two β -thalassemia patients. As with autologous bone marrow transplantation, some of the patients' marrow cells were cryopreserved as a backup. The lentiviral vector particles containing a functional β -globin were

introduced into the remaining cells. After the transfected cells were cultured for one week *ex vivo*, some were also cryopreserved. The patients were conditioned with intravenous busulfan (3.2 mg/kg/day for four days) without the addition of cyclophosphamide, before transplantation using the autologous gene-modified cryopreserved cells (Fig. 7) [34].

The first patient failed to engraft because the HSCs had been compromised by how they were handled, not because of any issues with the gene therapy vector, and ultimately used backup bone marrow. The second patient, as described previously, achieved long-term β -globin production; one-third of the patient's hemoglobin was produced by the genetically modified cells [33].

Furthermore, the detailed examination of the transgenic cells showed significantly increased expression of high mobility group AT-hook 2 (HMGA2), which interacts with transcription factors to regulate gene expression, in the clones where gene insertion occurred in the *HMGA2* gene. The proportion of the HMGA2 overexpressing clones increased with time, to over 50% of transgenic cells at 20 months after gene therapy. In this patient, the HMGA2 overexpressing cells were only 5% of all circulating hematopoietic cells and there was no evidence of malignant transformation. However, researchers point out that there was expressive production of a truncated form of the HMGA2 protein. Since truncated or overexpressed HMGA2 is observed with some blood cancers and non-malignant expansions of blood cells, caution is recommended with this therapy [34].



Figure 7. Gene-therapy procedure for patient with b-thalassemia. a. Hematopoietic stem cells (HSCs) are collected from the bone marrow of a patient with β -thalassemia and maintained them in culture. b, Lentiviral-vector particles containing a functional β -globin gene were then introduced into the cells and allowed them to expand further in culture. c. To eradicate the patient's remaining HSCs and make room for the geneticaaly modified cells, the patient underwent chemotherapy. d. The genetically modified HSCs were then transplanted into the patient (Reproduced from [34]).

Recently, researchers generated a LCR-free SIN lentiviral vector that combines two hereditary persistence of fetal hemoglobin (HPFH)-activating elements, resulting in therapeutic levels of A γ -globin protein produced by erythroid progenitors derived from thalassemic HSCs [35]. Both lentiviral-mediated γ -globin gene addition and genetic reactivation of endogenous γ -globin genes are considered potentially capable of providing therapeutic levels of hemoglobin F to patients with β -globin deficiency [36]. In addition, a trial of γ -globin induction with β -globin production using mithramycin, an inducer of γ -globin expression, to remove excess α -globin proteins in β -thalassemic erythroid progenitor cells was reported [37].

4.2. Fanconi anemia

Fanconi anemia is a hereditary disease characterized by cellular hypersensitivity to DNA crosslinking agents. It leads to bone marrow failure, such as aplastic anemia, by approximately eight years of age. Since there is a high risk of developing malignancy, HSCT has been performed as a curative treatment for bone marrow insufficiency. Although the tenyear probability of survival after transplant from an Human leukocyte antigen (HLA) -identical donor is over 80%, results with other donors are not satisfactory. HSC gene therapy is considered an alternative in cases where there is no HLA-identical donor available [38-40].

There are currently 13 discovered Fanconi anemia complement groups and 13 distinct genes (FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN) have been cloned. Mutations in FANCB are associated with an Xlinked form of Fanconi anemia; mutations in the other genes are associated with autosomal recessive transmission. Although frequencies vary by geographical region, FANCA gene abnormalities are found in more than half of all Fanconi anemia patients [41]. Although one of the major hurdles in the development of gene therapy for Fanconi anemia is the increased sensitivity of Fanconi anemia stem cells to free radical-induced DNA damage during ex vivo culture and manipulation, retroviral and lentiviral vectors have been successfully employed to deliver complementing Fanconi anemia cDNA to HSCs with targeted disruptions of the FANCA and FANCC genes [20, 42-44]. In a phase I trial of FANCA gene therapy, gene transfer was performed with patient bone marrow-derived CD34⁺ cells and the MSCV retroviral vector [38]. Whether sufficient HSCs can be obtained is a potential problem in Fanconi anemia patients due to possible bone marrow insufficiency, but in this study, sufficient target CD34⁺ cells were obtained from most patients. Two patients had FANCA-transduced cells successfully infused. The procedure was safe, well tolerated, and resulted in transient improvements in hemoglobin and platelet counts [39]. However, transduced cell products were not obtained in one patient who required cryopreserved bone marrow. The first clinical study of FANCC gene therapy using a retroviral vector involved four patients. Although functional FANCC gene expression was observed in peripheral blood and bone marrow cells, the results were transient [43].

Engraftment efficiency of *FANCA*-modified cells using a lentiviral vector was studied in a mouse model. Rapid transduction with four hours of culture using only SCF and megakaryocyte growth and development factor and minimal differentiation of gene-induced cells is better than standard 96-hour culture using a variety of cytokines, including SCF, interleukin-11, Flt-3 ligand, and IL-3 [44]. Moreover, a recent trial demonstrated enhanced viability and engraftment of gene-corrected cells in patients with *FANCA* abnormalities with short transduction (overnight), low oxidative stress (5% oxygen), and the anti-oxidant N-acetyl-Lcysteine [20]. Lentiviral transduction of unselected Fanconi anemia bone marrow cells mediates efficient phenotypic correction of hematopoietic progenitor cells and CD34⁻ mesenchymal stromal cells, with increased efficacy in hematopoietic engraftment [45]. In *Fancg -/-* mice, the wild-type mesenchymal stem and progenitor cells play important roles in the reconstitution of exogenous HSCs *in vitro* [46]. Recently, a new approach that directly injects lentiviral vector particles into the femur for *FANCC* gene transfer in mice was able to successfully introduce the *FANCC* gene to HSCs. This result provides evidence that targeting the HSCs directly in their native environment enables efficient and long-term correction of bone marrow defects in Fanconi anemia [47].

In recent years, the design of lentiviral vectors used for gene therapy in Fanconi anemia has improved. Although the vav and phosphoglycerate kinase (PGK) promoters are relatively weak, physiological levels of *FANCA* gene expression can be obtained in lymphoblastoid cells. CMV and spleen focus-forming virus (SFFV) promoters result in overexpression of *FANCA*. The PGK-*FANCA* lentiviral vectors with either a wild-type woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) or a mutated WPRE in the 3' region have higher levels of *FANCA* gene expression. In conclusion, lentiviral vectors with a mutated WPRE and a PGK promoter are considered the most suitable with respect to safety and efficiency for Fanconi anemia gene therapy [48].

There was a recent interesting report on the use of induced pluripotent stem cells (iPS cell). Instead of introducing a repaired gene into the HSCs of a patient with a *FANCA* gene abnormality, the modified gene was introduced into more stable somatic cells, e.g. fibroblasts, and iPS cells were derived from the genetically modified somatic cells. If HSCs can be produced from genetically modified iPS cells, hematological function can be efficiently reconstructed in patients with hematologic disorders [49].

4.3. Hemophilia

Hemophilia is a common congenital coagulopathy caused by coagulation factor VIII (hemophilia A) or IX (hemophilia B) deficiency. Although the genes encoding both factor VIII (Xq28) and factor IX (Xq27) are located on the X chromosome and most cases are X-linked, many sporadic variations have been reported. Factor substitution therapies have been used to treat hemophilia for many years. However, there is great hope for gene therapy with hemophilia because coagulation factors have short half-lives (factor VIII, 8 to 12 hours; factor IX, 18 to 24 hours), and an inhibitor is produced in many cases. Furthermore, it is possible for gene therapy to suppress immunogenicity by introducing a mutant protein that lacks the domain with which the inhibitor interacts. Since both coagulation factors are usually produced in the liver, there are few studies involving HSCs. In addition to hepatocytes, trials introducing the modified gene directly into splenic cells, endothelial cells, myoblasts, fibroblasts, etc. have been reported [50-52]. Since the factor IX gene (34 kb) is smaller than the factor VIII gene (186 kb), hemophilia B gene therapy can be possible with an adenovirus vector or an AAV vector. Therefore, hemophilia B is progressing more as a field of gene therapy research even through there are five times more patients with hemophilia A [51-53].

Recently, human factor VIII variant genes were successfully introduced into the HSCs of a mouse with hemophilia A resulting in therapeutic levels of factor VIII variant protein expression. This variant factor VIII has changes in the B and A2 domain in addition to the A1 domain for improved secretion and reduced immunogenicity (wild-type factor VIII has six domains, A1, A2, B, A3, C1, and C2) [54]. To ameliorate the symptoms of hemophilia A, partial replacement of the mutated liver cells by healthy cells in hemophilia A mice was challenged with allogeneic bone marrow progenitor cell transplantation. In this study, the bone marrow progenitor cell-derived hepatocytes and sinusoidal endothelial cells synthesized factor VIII, showing that autologous gene-modified bone marrow progenitor cells have the potential to treat hemophilia [55].

4.4. Primary immunodeficiencies

Although HSCT has been widely performed as curative treatment for primary immunodeficiencies, gene therapy has been considered when there is no HLA-identical donor available. As previously shown, the first successful gene therapy was performed in a patient with ADA deficiency in the U.S. in 1990. Since the gene was introduced into T lymphocytes, frequent treatment was required. However, this treatment was associated with an unacceptable level of toxicity. Since transfected vector and normal ADA gene expression in T lymphocytes continued for two years after the cessation of treatment [1], gene therapy attracted attention. With advances in HSC gene-transfer technology, gene therapy for many primary immunodeficiencies can now be considered [56].

4.4.1. SCID-X1

SCID-X1 is an X-linked disease caused by deficiency of the common γ (γ c) chain in the IL-2 receptor. Because the γ c chain is common to the IL-4, IL-7, IL-9, IL-15, and IL-21 receptors, in SCID-X1 patients, there are defects in T and natural killer (NK) cells, and B cell dysfunction are usually observed [57]. Patients begin suffering from various infections starting several weeks after birth. Without curative treatment, such as HSCT, patients die in infancy.

In SCID-X1, since T cells are lacking, engraftment of the gene-transduced cells can be achieved without pre-conditioning therapy. In the clinical studies of SCID-X1 patients in France and the U.K., the MFG retroviral vector was used with HSCs obtained from the patient. After gene therapy, many patients had improvements in immune function. However, since the genes regulating lymphocyte proliferation, such as *LIM domain only 2 (LMO2)*, *Bmi1*, *cyclin D2 (CCND2)* are near the gene insertion region, there was a high frequency of T-cell leukemia after treatment. Furthermore, in the patients who developed leukemia, additional chromosomal changes, including activating mutations of *Notch1*, changes in the T cell receptor β region, and deletion of tumor suppressor genes, e.g. cyclin-dependent kinase-2A (*CDKN2A*) were observed [58]. Almost gene integration sites by the retroviral vector were inside or near genes that are highly expressed in CD34 positive stem cells. Furthermore, the activity of protein kinases or transferases coded by these activated genes was stronger in CD3 positive T cells than CD34 positive cells [59]. Thus, gene integration mediated by a retrovirus influences the target cell's dormant capacity for survival, engraftment, and proliferation. Although continuous T cell production was founded in many cases, there was little reconstruction of myeloid cells and B cells, and some patients required continuous immunoglobulin substitution therapy. The use of conditioning therapy is also related to immunological reconstruction after γc chain gene therapy. There is decreased NK cell reconstruction without conditioning therapy, so conditioning chemotherapy is required for the engraftment of undifferentiated stem cells [58]. A trial of SCID-X1 gene therapy in the U.S. involved three patients ranging from 10 to 14 years of age. They had poor immunological recovery after allergenic HSCT and T cell recovery was only observed in the youngest patient, suggesting there is a limit to the recovery of the function of the thymus in older children [60].

To study whether activation of genes near the region of gene insertion or inserted γc chain gene expression itself induces oncogenicity during SCID-X1 gene therapy, a study of the human γc chain gene being expressed under the control of the human CD2 promoter and LTR (CD2- γc chain gene) was performed in mice. When the CD2- γc chain gene was expressed in transgenic mice, a few abnormalities involving T cells were observed, but tumorigenesis was not observed and T and B cell functions were recovered in γc chain-gene deficient mice. This study demonstrated that when the γc • c chain gene is expressed externally, SCID-X1 may be treated safely [61].

Although SIN vectors were developed from earlier retroviral [62] or lentiviral vectors [63] to reduce the risk of oncogenicity in SCID-X1 gene therapy, genotoxicity unrelated to mutations in gene insertion regions or γc chain gene overexpression have been reported with lentiviral vectors in recent years, and it seems that more sophisticated vector development is required [64].

4.4.2. ADA-SCID

ADA is an enzyme that catalyzes the conversion of purine metabolism products adenosine and deoxyadenosine into inosine or deoxyinosine. ADA-SCID is an autosomal recessive disease that results in the accumulation of adenosine, deoxyadenosine, and deoxyadenosinetriphosphate (dATP). Accumulated phosphorylated purine metabolism products act on the thymus and cause the maturational or functional disorder of lymphocytes. Because ADA-SCID patients have both T and B cell production fail, patients have a severe combined immunodeficiency disease with a clinical presentation similar to SCID-X1 results, but unlike SCID-X1, many patients have a low level of T cells. Although enzyme replacement therapy with polyethylene glycol–modified bovine ADA (PEG-ADA) was developed to treat ADA-SCID, it is limited by the development of neutralizing antibodies and the cost of lifelong treatment.

In ADA-SCID, since T cell counts are increased by PEG-ADA, gene therapy to increase peripheral T cell counts was attempted during the early stages of gene therapy. Although adverse events were not observed and continuous expression of ADA was achieved in many patients, reconstruction of immune function was not obtained and substitution therapy with PEG-ADA remained necessary. Therefore, HSCs were no longer the target of gene therapy for ADA-SCID. Since ADA-SCID patients have T cells, nonmyeloablative conditioning was performed to achieve gene-transduced HSC engraftment [25, 65].

In a joint Italian-Israeli study started in 2000, ten ADA-SCID children were infused with CD34 positive cells transduced with a MoMLV retroviral vector containing the *ADA* gene after nonmyeloablative conditioning with busulfan (2mg/kg/day for two days). T cell counts or function were improved in nine out of the ten patients, and PEG-ADA was discontinued in eight. Many patients also had improvements in B or NK cell function, and immunoglobulin substitution therapy was discontinued in five patients. Although some patients had serious adverse events including prolonged neutropenia, hypertension, Epstein-Barr virus infection, and autoimmune hepatitis, there were no cases of treatment-induced leukemia [25].

As with SCID-X1, the retroviral vector gene insertion region is also near genes that control cell proliferation or self-duplication, such as *LMO2*, or proto-oncogenes [66]. In clinical studies performed in France, the U.S., and the U.K., none of the ADA-SCID patients had adverse events related to insertional mutagenesis, such as leukemia [67, 68]. Thus, HSC gene therapy for ADA-SCID using a lentiviral vector [69] is expected to become the alternative therapy in cases without a suitable donor for HSCT [70]. As an alternative to HSC-based gene therapy, a study using an AAV vector has reported ADA gene expression in various tissues, including heart, skeletal muscle, and kidney [71].

4.4.3. CGD

CGD is a disease caused by an abnormality in nicotinamide dinucleotide phosphate (NADPH) oxidase expressed in phagocytes, resulting in failure to produce reactive oxygen species and decreased ability to kill bacteria or fungi after phagocytosis. NADPH oxidase consists of gp91^{phox} (Nox2) and p22 ^{phox} which together constitute the membrane-spanning component flavocytochrome b558 (CYBB), and the cytosolic components p47^{phox}, p67^{phox}, p40^{phox}, and Rac. CGD is caused by a functional abnormality in any of these components. Mutations in *gp91^{phox}* on the X chromosome account for approximately 70% of CGD cases. CGD patients are afflicted with recurrent opportunistic bacterial and fungal infections, leading to the formation of chronic granulomas. Although lifelong antibiotic prophylaxis reduces the incidence of infections, the overall annual mortality rate remains high (2%–5%) and the success rate of HSCT is limited by graft-versus-host-disease and inflammatory flareups at infected sites [56].

In the initial trials of CGD gene therapy without any conditioning therapy, *p*47^{*phox*} or *gp*91^{*phox*} gene was inserted using a retroviral vector. The inserted gene was expressed in peripheral blood granulocytes three to six weeks after re-infusion and mobilization by granulocyte colony-stimulating factor (G-CSF), but there was no clinical effect within six months [72-74].

In a German study where *gp91phox* was inserted with busulfan conditioning (8mg/kg), there were fewer infections after gene therapy. Gene expression was observed in 20% of leukocytes in the first month, rising to 80% at one year. However, in the gene insertion region there are genes related to myeloid cell proliferation, such as *myelodysplastic syndrome 1-ecotropic virus integration site 1 (MDS1/EVI1), PR domain containing protein 16 (PRDM16), SET binding protein 1 (SETBP1).* Two patients developed myelodysplasia [75]. These two patients had monosomy 7, considered to be related to *EVI1* activation. One died of severe sepsis 27

months after gene therapy. Although the gene-inserted cells remained expressed in this patient, methylation of the CpG site in the LTR of the viral vector was observed and the expression of the inserted $gp91^{phox}$ gene was decreased. Interestingly, methylation was restricted to the promoter region of the LTR; the enhancer region was not methylated. Therefore, although $gp91^{phox}$ gene expression was decreased, the activation of *EVI1* near the inserted region occurred, leading to clonal proliferation [76]. Since there is a possibility that the transcription activity of genes related to myeloid cell proliferation near the gene insertion site will be increased, there remains a concern about tumorigenesis with peripheral stem cells mobilization by G-CSF in CGD patients, as with X-SCID [74].

Recently, next-generation gene therapy for CGD using lineage- and stage-restricted lentiviral vectors to avoid tumorigenesis [77] and novel approaches involving iPSs derived from CGD patients using zinc finger nuclease (ZFN)-mediated gene targeting were studied [78]. Specific gene targeting can be performed in human iPSs using ZFNs to induce sequence-specific double-strand DNA breaks that enhance site-specific homologous recombination. A single-copy of $gp91^{phox}$ was targeted into one allele of the "safe harbor" AAVS1 locus in iPSs [79].

4.4.4. Wiskott-Aldrich Syndrome (WAS)

WAS is a severe X-linked immunodeficiency caused by mutations in the gene encoding the WAS protein (WASP), a key regulator of signaling and cytoskeletal reorganization in hematopoietic cells. Mutations in *WAS* gene result in a wide spectrum of clinical manifestations ranging from relatively mild X-linked thrombocytopenia to the classic WAS phenotype characterized by thrombocytopenia, immunodeficiency, eczema, high susceptibility to developing tumors, and autoimmune manifestations [80]. Preclinical and clinical evidence suggest that WASP-expressing cells have a proliferative or survival advantage over WASP-deficient cells, supporting the development of gene therapy [56]. Furthermore, up to 11% of WAS patients have somatic mosaicism due to spontaneous *in vivo* reversion to the normal genotype, and in WAS patients, accumulation of normal T-cell precursors are sometimes seen [81].

In one preclinical study introducing the *WAS* gene into human T and B cells or mouse HSCs using a retroviral vector, recovery of T cell function and immune reactions to infection were observed [82, 83]. The first clinical study of WAS using HSCs involved two young boys in Germany. The WASP-expressing retroviral vector was transfected into CD34 positive cells obtained by apheresis of peripheral blood. Busulfan was used for conditioning therapy (4mg/kg/day for two days). Over two years, WASP gene expression by HSCs, lymphoid and myeloid cells, and platelets was sustained, and the number and function of monocytes, T, B, and NK cells normalized. Clinically, hemorrhagic diathesis, eczema, autoimmunity, and the predisposition to severe infections were diminished. Since comprehensive insertion-site analysis showed vector integration near multiple genes controlling growth and immunologic responses in a persistently polyclonal hematopoiesis, careful monitoring for tumorigenesis is necessary, as with SCID-X1 and CGD [84, 85].

SIN lentiviral vectors using the minimal domain of the WAS promoter or other ubiquitous promoters, such as the PGK promoter, are currently being developed for WAS gene therapy. Preclinical studies using the HSCs obtained from mice or human patients have yield good results in terms of gene expression and genotoxicity [86-90].

Since a study using human embryonic stem cells (hESCs) and WAS-promoter–driven lentiviral vectors labeled by green fluorescent protein (GFP) showed highly specific gene expression in hESCs-derived HSCs, the WAS promoter will be used specifically in the generation of hESC-derived HSCs [91].

4.4.5. Janus Kinase 3 (JAK3) deficiency

JAK3 deficiency is characterized by the absence of T and NK cells and impaired function of B cells, similar to SCID-X1. Treatment consists of HSCT with an HLA-identical or HLA-haplo-identical donor, often the parents of the patient, with T cell depletion. Engraftment is successful in most cases.

Although the recovery of T cell function is usually observed after HSCT, there are usually no improvements in B or NK cell function [92]. One case report involved introduction of *JAK3* into the patient's bone marrow CD34 positive cells using the MSCV retroviral vector. In this study, immunological recovery was not achieved although gene expression was observed for seven months [93]. Since JAK activation can cause T-cell lymphoma, tumorigenesis remains a concern with JAK gene therapy [92].

4.4.6. Purine Nucleoside Phosphorylase (PNP) deficiency

PNP metabolizes adenosine into adenine, inosine into hypoxanthine, and guanosine into guanine. PNP deficiency is an autosomal recessive metabolic disorder characterized by le-thal T cell defects resulting from the accumulation of products from purine metabolism.

In PNP-deficient mice, transplantation of bone marrow cells transduced with a lentiviral vector containing human *PNP* resulted in human PNP expression, improved thymocyte maturation, increased weight gain, and extended survival. However, 12 weeks after transplant, the benefit of *PNP*-transduced cells and the percentage of engrafted cells decreased [94].

4.4.7. Leukocyte Adhesion Deficiency type 1 (LAD-1)

LAD-1 is a primary immunodeficiency disease caused by abnormalities in the leukocyte integrin CD11/CD18 heterodimer due to mutations in the *CD18* gene. It is similar to canine leukocyte adhesion deficiency (CLAD). LAD-1 patients begin experiencing repeated serious bacterial infections immediately after birth.

In order to suppress gene activation near the gene insertion region in CLAD and to obtain the sufficient expression of the *CD18* gene, researches have used various promoters with a lentiviral vector or foamy virus, a retroviral vector. *In vivo* animal experiments using a PGK or an elongation factor 1α promoter did not lead to symptom improvement [95-97], but im-

provement was seen with CD11b and CD18 promoters, respectively, with a SIN lentiviral vector in one animal study [98].

4.5. Mucopolysaccharidosis (MPS)

MPS is a general term for diseases characterized by glycosaminoglycan (GAG) accumulation into lysosomes as a result of deficiencies in lysosomal enzymes that degrade GAG. Although there are more than ten enzymes that are known to degrade GAG, MPS is divided into seven types: type I (α -L-iduronidase deficiency, Hurler syndrome, Sheie syndrome, Hurler-Sheie syndrome), type II (iduronate sulfatase deficiency, Hunter syndrome), type III (heparan N-sulfatase deficiency, α -N-acetylglucosaminidase deficiency, α -glucosaminidase acetyltransferase deficiency, N-acetylglucosamine 6-sulfatase deficiency, Sanfilippo syndrome), type IV (galactose 6-sulfatase deficiency, Morquio syndrome), type VI (N-acetylgalactosamine 4-sulfatase deficiency, Maroteaux-Lamy syndrome), type VII (β -glucuronidase deficiency, Sly syndrome), and type IX (hyaluronidase deficiency). Type II is X-linked; the other types are autosomal recessive. Although lysosomes are found in almost all cells, MPS mainly affects internal organs such as the brain, heart, bones, joints, eyes, liver, and spleen. The extent of disease, including mental retardation, varies with MPS type.

In types I, II, and VI, enzyme replacement therapy is performed. HSCT is performed in types I, II, IV, and VII. Gene therapy for types I, II, III, and VII type have been investigated. There are trials using an AAV or adenovirus vector to insert the modified gene into various cell types, including hepatocytes, muscle cells, myoblasts, and fibroblasts [99].

The first study of HSC gene therapy for MPS using a retroviral vector was performed on type VII mice in 1992, resulting in decreased accumulation of GAG in the liver and spleen but not in the brain and eyes [100]. Subsequent studies in type I and III animal models showed decreases in GAG accumulation in the kidneys and brain. Introductory efficiency and immunological reactions are considered challenges in HSC gene therapy for MPS [99].

Restoring or preserving central nervous system (CNS) function is one of the major challenges in the treatment of MPS. Since replaced enzymes easily cannot pass the bloodbrain barrier (BBB), a high dose of enzyme is needed to improve CNS function. Gene therapy faces the same challenge. Even with high expression of enzyme by, for example, hepatocytes, the BBB prevents efficient delivery into the CNS. When a lentiviral vector is directly injected into the body, gene expression in brain tissue is observed, although the underlying mechanism is unknown. There are also trials where AAV vectors are directly injected into the CNS of mice or dogs and gene expression was observed in brain tissue [99].

Recently, a lentiviral vector using an ankyrin-1-based erythroid-specific hybrid promoter/ enhancer (IHK) was used with HSCs to obtain gene expression only in erythroblasts for type I MPS. This approach resulted in decreased accumulation of GAG in the liver, spleen, heart, and CNS via enzyme expression in erythroblasts [101].

4.6. Gaucher disease

Gaucher disease is the most common lysosomal storage disorder. It is caused by deficiency of glucocerebroside-cleaving enzyme (β -glucocerebrosidase), resulting in the accumulation of glucocerebroside in the reticuloendothelial system [102]. This autosomal recessive disease presents with hepatosplenomegaly, anemia, thrombocytopenia, and convulsions with or without mental retardation. It is classified into three types based on the clinical course or existence of neurological symptoms: type I (non-neuropathic, adult type), type II (acute neuropathic, infantile type), and type III (chronic neuropathic, juvenile type). Enzyme replacement therapy has been established in type I. As with MPS, since it is difficult to improve CNS symptoms with enzyme replacement therapy is considered in cases with little improvement with enzyme replacement therapy [103].

For Gaucher disease without CNS symptoms, a animal model using an AAV vector to produce enzyme in hepatocytes yielded good results [103]. HSC gene therapy using a retroviral vector was attempted in type I mice. The treated cells had higher β -glucocerebrosidase activity than the HSCs from wild-type mice. Glucocerebroside levels normalized five to six months after treatment and no infiltration of Gaucher cells could be observed in the bone marrow, spleen, and liver [104]. In recent years, development of lentiviral vectors including the human glucocerebrosidase gene [105] and low-risk HSCT with nonmyeloablative doses of busulfan (25mg/kg) and no radiation therapy have been attempted in mice [106].

4.7. X-ALD

X-ALD is a peroxisomal disease in which a lipid metabolism abnormality causes demyelination of CNS tissues and dysfunction of the adrenal gland. It results from mutations in the ATP-binding cassette sub-family D (*ABCD1*) gene that codes for the adrenoleukodystrophy (ALD) protein. Behavioral disorders, mental retardation, or both occur by the age of five or six. Once symptoms appear, they progress to gait disorder and visual impairment within several months and the prognosis is poor. Increased levels of very long chain fatty acids (VLCFA), such as C25:0 or C26:0, are observed in the CNS, plasma, erythrocytes, leucocytes, etc. If the neurological defects are not severe, arrest of or improvement in symptoms can be obtained with HSCT [107].

One study has reported the introduction of wild-type *ABCD1* using a lentiviral vector into peripheral blood CD34 positive cells of two patients with no HLA-identical donor. The patients received a transfusion of autologous gene-modified cells after myeloablative conditioning therapy. At three years of follow-up, ALD proteins were expressed in approximately 7–14% of neutrophils, monocytes, and T cells. Clinically, cerebral demyelination stopped 14 and 16 months after gene therapy, respectively, similar to results with allergenic HSCT [108, 109].

5. Conclusion

Gene therapy using HSCs was outlined. HSCT with HSCs can replace all of the patient's original HSCs with donor HSCs. Therefore, gene therapy using HSCs is an alternative if the

patient does not have an HLA-identical donor or cannot tolerate the conditioning regimen or other HSCT-related side effects. Fully myeloablative or nonmyeloablative conditioning regimens are still necessary to eliminate potential competition within the bone marrow compartment, in an attempt to increase the number of gene-modified HSCs or progenitors that produce the therapeutic enzyme or protein. With gene therapy, eliminating the risk of immune reactions against the transgene is necessary. Lentiviral vectors in clinical use must not be contaminated by replication-competent recombinant vectors related to the parent HIV-1 virus. The main risk of retrovirus- or lentivirus-mediated gene therapy may prove to be insertional mutagenesis caused by random retroviral integration leading to activation of proto-oncogenes or inactivation of tumor-suppressor genes, ultimately leading to malignancy [107]. However, with advances in gene introduction technology, such as the development of the SIN vector and advances in cell or gene-region targeting, gene therapy can be done more safely and efficiently. Furthermore, since cells more immature than HSCs, i.e., iPS cells, are available, further advances in HSC gene therapy are expected in the future.

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