ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info/jasr

ISSN
0976-9595
Review Article

Lentiviral Mediated Correction of Genetic Disorders

Suparna Dugal *, Aasia Choudhary

Dept.of Microbiology, Sophia College, University of Mumbai, Maharashtra, India.

**Corresponding author: suparnadugal@gmail.com*

ABSTRACT

Recently lentiviral vectors (LVs) have emerged as potent and versatile vectors for gene transfer into cells. Use of LVs has now moved beyond the preclinical stage into the clinical arena with multiple gene therapy trials ongoing or approved for the treatment of genetic/ metabolic disorders. LVs can deliver genes *ex vivo* into bona fide stem cells, particularly hematopoietic stem cells, allowing for stable transgene expression upon hematopoietic reconstitution. In the last decade, gene therapy has established itself as a promising approach for the treatment of hereditary diseases of the blood-forming system, especially for patients who cannot be treated by conventional transplantation strategies. However, despite these advances, for many envisioned applications of lentivirus vectors as tools in biology and therapeutic gene delivery in the future, the efficiency of gene transfer still needs to be further studied and improved. Additionally, there are safety concerns regarding insertional mutagenesis. The focus of this review is to highlight some important investigations in which lentiviral vector technology platform was employed to provide a robust ammunition in the quest to develop improved therapeutics to treat otherwise incurable genetic diseases like glycogen storage disease type Ia, β-thalassemias and X linked severe combined immunodeficiency syndrome.

Keywords: Lentiviral vectors, glycogen storage disease type Ia, β- thalassemia, lysosomal storage disorders, Fanconi anemia, SCID-X1.

1. INTRODUCTION

Gene therapy can be broadly defined as the treatment of a disease or medical disorder by the introduction of therapeutic genes into the appropriate cellular targets. These therapeutic genes can correct deleterious consequences of specific gene mutations or re-programme cell functions to overcome a disease. For successful gene therapy, the exogenous therapeutic gene has to be specifically, efficiently and stably incorporated into the target cell.

Lentivirus is a genus of the Retroviridae family, characterized by long incubation period [1]. Lentiviral vectors (LVs) have become some of the most widely used vectors for fundamental biological research, functional genomics and gene therapy. LV resembles γ-retroviral vectors (γ-RVs) in their ability to stably integrate into the target cell genome, resulting in persistent expression of the gene of interest. However, in contrast to γ-RVs, LV can also transduce nondividing cells. This distinctive feature paves the way towards many applications for which γ-RVs are not suitable. The lack of pre-existing immunity to vector components in most subjects may give LVs a possible advantage over other vector systems such as AAV or adenovirus. Moreover, LVs can accommodate larger transgenes [upto 10 kilobases (kb)] compared to when γ-RVs are used, though vector titre tends to decrease with larger inserts. Examples of lentiviruses include HIV, SIV and FIV [2].

The current review focuses on the advances made and clinical trials done in gene therapy for genetic disorders like β-thalassemias, X-linked severe combined immunodeficiency and Fanconi anemia. The reported success of some recent clinical trials using lentiviral vectors allows hope of bringing this technology into medical reality.

2. GLYCOGEN STORAGE DISEASE TYPE Ia

Glycogen storage disease type Ia (GSD-Ia or von Gierke disease) is an autosomal recessive disorder caused by a deficiency in glucose-6-phosphatase-α (G6Pase-α or G6PC) [3] that catalyzes the hydrolysis of glucose-6-phosphate to glucose and phosphate in the terminal step of gluconeogenesis and glycogenolysis. GSD-Ia patients manifest a phenotype of disturbed glucose homeostasis characterized by fasting hypoglycemia, hepatomegaly, nephromegaly, hypercholesterolemia, hypertriglyceridemia, hyperuricemia, lactic acidemia, and growth retardation .Current treatment of GSD-Ia involves continuous infusion of glucose or frequent oral administration of uncooked cornstarch in order to control the symptomatic hypoglycemia; however, these regimens do not cure the disease and long-term complications commonly develop [4]. Moreover, efficacy of the dietary therapy is frequently limited due to poor compliance.

Gene therapy using lentivirus (LV) and adeno associated viral (AAV) vectors has been proposed as a possible option for treatment of this illness [1, 4, 6]. A detailed characterization of the molecular origins of GSDIa allowed for the creation and recognition of GSDIa animal models. Dr Janice Chou created the G6Pase-knockout (KO) mouse in 1993, and this strain has since been widely used as a gold standard for studying GSDIa in the laboratory [5]. Although a naturally occurring dog model for G6Pase-α deficiency also exists, the long lifespan and high level of husbandry required for maintaining these animals has resulted in continued interest in the mouse model. Both lentivirus and adeno associated virus mediated gene therapies have been evaluated for the disease in these model systems [5]. Genetically engineered *G6Pase* knockout (KO) mice and canine colonies carrying a naturally occurring *G6Pase* mutation both exhibit disease symptoms similar to human patients and enable the testing of diverse therapeutic approaches to the disease. G6Pase-deficient mice survive, at most, a few weeks even upon daily administration of glucose. A recent report describes a specific husbandry protocol that allowed ~60% of KO mice to withstand weaning and live to adulthood; however, they required extensive palliative care, and the liver histology and glycogen accumulation did not improve with age [1, 5].

In the study done by Koeberl *et al*, an adeno associated virus vector containing the canine G6Pase promoter driving canine complementary DNA(cDNA) expression prolonged survival to 28 weeks in G6Pase (-/-) mice, when pseudotyped as AAV8 (AAV2/8) and administered at 2 weeks of age. The AAV2/1 vector improved hypoglycemia and prolonged the survival of G6Pase (-/-) mice, when neonates were treated with two injections 1 week apart. Neither the above mentioned AAV2/1 nor AAV2/8 vector completely corrected blood glucose level to those of wild type littermates, and both were administered at very high particle number ($\sim 2X10^{14}$ vector particles (vp)/kg body weight). By contrast, a helper dependent adenoviral vector encoding canine G6Pase completely corrected hypoglycemia during fasting, but failed to achieve uniform long term survival of infant G6Pase (-/-) mice. Both AAV and helper dependent adenoviral vectors failed to fully correct G6Pase deficiency in the liver of G6Pase (-/-) mice, indicating the need for higher transgene expression in GSD-Ia [6, 7].

Adeno-associated viral vectors integrate into the host cell genome at very low frequencies and remain primarily in an episomal form, thus providing prolonged transgene expression only in the absence of cell division. In human trials, these vectors were shown to induce an immune response resulting in the production of neutralizing antibodies that led to elimination of the transduced cells. In contrast to adeno-associated virus, lentiviral vectors efficiently integrate into the cellular genome, providing long-term expression even in cells that are actively dividing. Furthermore, an immune response is not elicited to vector components, even upon repeated viral administration. Due to safety concerns and public fear of human immunodeficiency virus–based delivery systems, vectors were developed from feline immunodeficiency virus (FIV), a nonprimate lentivirus that infects cats and is phylogenetically only distantly related to the primate lentiviruses. Despite repeated exposure to FIV, neither seroconversion nor other evidence of infection in humans was observed, mainly due to inefficient replication of the virus in human cells owing to the negligible transcriptional activity of the FIV long-terminal repeat (LTR).

The FIV vector used in the study done by A. Grinshpun *et al* was deleted of the enhancer located in the U3 region of the 3′LTR, thus ensuring self-inactivation following transduction (self-inactivating vector) [1]. In addition, it contained a mutated woodchuck hepatitis post-transcriptional regulatory element (WPRE) located downstream of the transgene, which served to enhance transgene expression and prevent transcription read-through into cellular genes. The mutation in WPRE ablated the promoter and the translation initiation site of the downstream woodchuck X-protein and thus prevented its potential oncogenic activity. The study consisted of 3 major steps- expression and biodistribution of reporter genes in FIV-transduced neonates, expression of human G6Pase in liver and kidney cell lines following transduction with an FIV-based vector and alleviation of disease symptoms in G6Pase-α KO mice following neonatal transduction. The viral transduction of neonates resulted in progressively normalized blood glucose levels that significantly prolonged their life span. Furthermore, serum lipid profiles, liver glycogen storage, and body weight were markedly improved, although kidneys and livers remained enlarged. Although the studies were limited to 6 months, no tumor development was observed following transduction with FIV, a possible risk factor associated with integrating viral vectors .Single administration of FIV vectors containing the human *G6Pase* gene to G6Pase-$\alpha^{-/-}$ mice did not change the biochemical and pathological phenotype. However, a double neonatal administration protocol led to normalized blood glucose levels, significantly extended survival, improved body weight, and decreased accumulation of liver glycogen associated with the disease. Following a double FIV administration, liver histology revealed areas of normally appearing hepatocytes (Figure 1), as well as regions of cells bearing a high content of glycogen, possibly providing an explanation for the unresolved hepatomegaly. Regardless of the excess glycogen that was observed in the kidneys even after two viral administrations, hepatic G6Pase-α expression in the liver is sufficient to promote long-term survival in parallel with a substantial improvement in many clinical parameters [1].

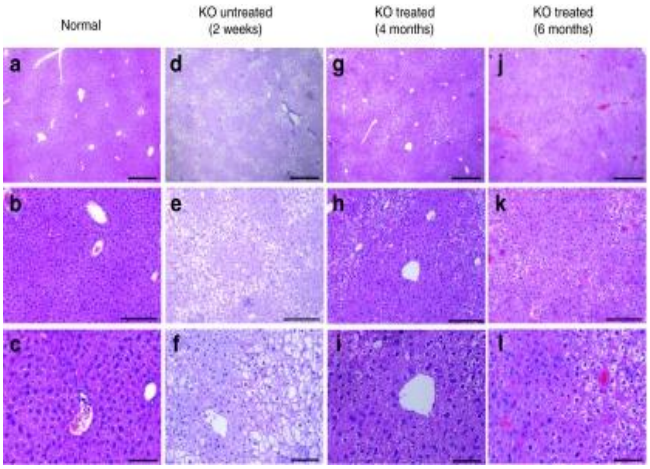

Fig. 1: Reduced lipid accumulation in the livers of G6Pase-α$^{-/-}$ mice following feline immunodeficiency virus vector administration. Representative photomicrographs of liver hematoxylin and eosin–stained sections from (a–c) normal, (d–f) untreated 2-week-old G6Pase-α KO pups, and treated G6Pase-α KO mice (g–i) 4 months and (j–l) 6 months after transduction. Original magnification ×40 in a,d, and g (bar = 100 μm); ×100 in b,e, and h (bar = 50 μm); ×200 in c, f, and i (bar = 20 μm). KO, knockout.

One limitation of the gene therapy approach is the possible induction of an immune response to the therapeutic protein or vector components, which would limit the efficacy and long-term duration of the treatment but studies have shown also that gene therapy administered at the neonatal stage minimizes immune response to the transgene as well as to the FIV vector. Hence neonatal gene therapy using Lentiviral vectors is an attractive treatment approach for early-onset inherited hepatic diseases in general and GSD-Ia in particular [1, 2, 5].

3. β – THALASSEMIAS

Thalassemias are a heterogeneous group of inherited anemias that collectively represent the most common monogenic disorders. The β-thalassemias are characterized by reduced or absent production of hemoglobin β-chains. The most severe form, β-thalassemia major or Cooley's anemia is characterized by a profound anemia that, if not treated, leads to death in the first year of life. The only available cure is allogeneic bone marrow (BM) transplantation which is, however, available for less than 30% of patients [8]. Autologous transplantation of genetically corrected hematopoietic stem cells (HSCs) is considered an attractive therapeutic alternative for patients lacking a compatible donor [9]. The development of LVs and the optimization of HSC transduction conditions has led to the recent application of LVs expressing the human β-globin gene in preclinical murine models and in human thalassemic cells [10-14].

Hematopoietic stem cell targeted gene therapy is an attractive approach for several hematopoietic disorders caused by single gene defects [10]. Gene therapy for β-thalassemia requires stable transfer of a β-globin gene into hematopoietic stem cells (HSCs) and high and regulated hemoglobin expression in the erythroblastic progeny [9]. In the context of gene therapy for thalassemia, the minimal dose of transduced HSCs required to correct the phenotype and the potential selective advantage of the genetically corrected erythroblasts need to be determined. Survival advantage of genetically corrected erythroblasts has never been studied, and it is not predictable from BM transplantation experiments because the amount of β-globin produced by vector-transduced cells is unlikely to match that synthesized by normal cells. This issue is relevant to develop gene therapy protocols based on mildly myeloablative conditioning regimens [9, 10].

In the study done by Annarita Miccio *et al,* a minimal locus control region (LCR)-β-globin transcription unit containing a 2.7-kb fragment encompassing LCR elements HS2 and HS3, which was reported to have classical enhancer and dominant chromatin-opening functions, respectively was constructed [9]. This minimal LCR HS2/HS3 combination was linked to a fully functional mini-β-globin gene with 265 bp of 5′ and 300 bp of 3′ flanking sequences. The minimized LCR-β-globin transcription unit was inserted into a self-inactivating, HIV-derived LV in reverse orientation with respect to the 5′LTR-driven genomic transcript, generating the GLOBE LV.

The high viral titer of GLOBE and its molecular stability facilitate large-scale production and utilization at a clinical level. The therapeutic potential of GLOBE was evaluated in one of the most severe murine models of β-thalassemia intermedia, the *th3/+* mutant. Transplantation of LV-transduced HSCs into primary and secondary thalassemic recipients led to complete and persistent correction of anemia and hematological parameters, rescue of ineffective erythropoiesis, and correction of secondary organ associated pathology. Molecular analysis indicated that the engraftment of a finite number of transduced HSCs was sufficient to fully correct thalassemia, with an average vector copy number (VCN) of less than 1. Correction of thalassemia was also achieved in the murine model of Cooley's anemia. Transplantation of transduced β^0 fetal liver cells (FLCs) in lethally irradiated recipients resulted in all RBCs expressing chimeric human–murine Hb, rescue of all of the transplanted mice from lethality, reversion to a normal phenotype in half of the treated animals, and conversion to a thalassemia intermedia phenotype in the remaining ones. Long-term full chimeras had a VCN between 3 and 5. These values were not unexpected, considering that a consistent increase in Hb levels (from 3.5 to 13.4 g/dl) was necessary to correct this phenotype, and that human β-globin associates poorly with the murine α-chain, forming tetramers that might not be as functional as the murine ones. BM transplantation in thalassemia demonstrates that it is not necessary to completely ablate the patient's BM to obtain

clinical control of the disease. Transplanted patients with long-term, persistent mixed chimerism ranging from 20% to 50% are transfusion independent, allowing one to predict that partial replacement of thalassemic HSCs with genetically corrected cells would be sufficient, with no need for a full myeloablative regimen. In transplanted β-thalassemic mice, low-level chimerism (10–20%) resulted in a large majority of donor-derived RBCs, a significant increase in haemoglobin level, and diminished EMH. The proportion of donor erythroid progenitors/precursors (burst-forming units-erythroid or pro-erythroblasts) in BM of transplanted patients or mice paralleled the proportion observed for donor leukocytes, demonstrating that the amplification of the normal erythroid component does not occur up to the pro-erythroblast stage. However, data from BM transplantation cannot be directly translated to gene therapy because the amount of β-globin produced by vector-transduced cells was unlikely to be comparable to that synthesized by normal cells. Thus this demonstrates that correction of β-thalassemia is achieved by *in vivo* selection of genetically corrected erythroblastic progenitors, differentiating from a relatively limited number of transduced HSCs. These findings have relevant implications for the design of future clinical trials because they indicate that full myeloablation may not be necessary for gene therapy of thalassemia [9].

Despite improvements in HSC gene transfer protocols using retroviral vectors, levels of HSC-targeted gene transfer in nonhuman primate models and in human clinical marking protocols are significantly lower than those obtained in mice. Levels of genetically marked blood cells up to 5% to 10% using γ-retroviral vectors have been obtained in only a small number of human patients and long-term marking at these levels has not yet been reported. In a long-term study of primates transplanted with lentiviral vector-transduced $CD34^+$ cells, stable gene transfer levels of 3% to 12% 4 years after transplantation was observed. Levels of at least 15% and higher was needed for significant clinical benefit for SCD and β-thalassemia. In addition, if submyeloablative conditioning was used to reduce the regimen-related toxicity to patients, there would be a further reduction of the proportion of engrafting, genetically corrected cells resulting from dilution with residual endogenous HSCs. One approach to selectively enrich genetically corrected autologous HSCs to therapeutic levels was to incorporate a drug-resistance gene into the therapeutic vector. After transplantation, treatment with a stem cell toxic drug could then be used to enrich the drug-resistant, genetically modified HSCs while eradicating the endogenous, diseased HSCs [10, 11].

In a study, Huifen Zhao *et al,* developed a double gene lentiviral vector encoding both human γ-globin under the transcriptional control of erythroid regulatory elements and methylguanine methyltransferase (MGMT), driven by a constitutive cellular promoter [10]. MGMT expression provides cellular resistance to alkylator drugs, which can be administered to kill residual untransduced, diseased HSCs, whereas transduced cells are protected. Mice transplanted with β-thalassemic HSCs transduced with a γ-globin/MGMT vector initially had sub therapeutic levels of red cells expressing γ-globin. To enrich γ-globin–expressing cells, transplanted mice were treated with the alkylator agent 1,3-bis-chloroethyl-1-nitrosourea. This resulted in significant increases in the number of γ-globin–expressing red cells and the amount of fetal hemoglobin, leading to resolution of anemia. Selection of transduced HSCs was also obtained when cells were drug-treated before transplantation. Mice that received these cells demonstrated reconstitution with therapeutic levels of γ-globin–expressing cells. These data suggest that MGMT-based drug selection holds promise as a modality to improve gene therapy for β-thalassemia [10].

An important biosafety issue in approaching gene therapy for the hemoglobin disorders is whether globin regulatory elements in the context of lentiviral vectors can perturb the expression of endogenous genes. As globin gene therapy trials are formulated, it seems both prudent and warranted to perform further careful evaluations of globin vector design and biosafety using existing *in vitro* culture systems designed to assess functional genotoxicity caused by vector insertions, as well as available *in vivo* murine models of vector genotoxicity [15].

4. X-LINKED SEVERE COMBINED IMMUNODEFICIENCY (SCID-X1)

X-linked severe combined immunodeficiency (SCID-X1) is caused by mutations in the *IL2RG* gene that encodes the common γ-chain (γc), a functionally indispensable subunit of interleukin (IL) receptors 2, 4, 7, 9, 15, and 21[16,17]. Affected infants have profound defects in cellular and humoral immunity, typically lack T and natural killer (NK) cells, and have normal or elevated numbers of B cells that are unable to undergo immunoglobulin class switching and antibody production.[18,19]. Failure of T- and NK-cell ontogeny is thought to result from loss of signaling through the receptors for IL-7 and IL-15, respectively [20, 21]. The treatment of choice is bone marrow transplantation from a human leukocyte antigen–identical sibling donor. Most infants, however, lack a suitable donor and conventionally undergo a human leukocyte antigen–mismatched transplant that is associated with an increased risk of morbidity and mortality [22, 23]. In many infants, immunological reconstitution remains incomplete, particularly B-cell functions, with resultant lifelong requirement for immunoglobulin replacement therapy. Gene therapy offers these infants the potential for improved survival

rates and more complete immunological reconstitution without the risk of graft-versus-host disease.

Retroviral vector–mediated gene transfer into hematopoietic stem cells (HSCs) has become a useful and promising tool for treatment of life-threatening inherited hematologic disorders [24-29]. However, a significant risk of insertional mutagenesis has emerged as evidenced by 4 patients with X-linked severe combined immunodeficiency (SCID-X1) treated with a Moloney murine leukemia virus (MLV)–based vector developing clonal T-cell lymphoproliferation [29-33]. In 2 patients, the cause appeared to be at least in part due to MLV proviral vector integration either within or near the known T-cell proto-oncogene *LMO2*, which led to up-regulation of its expression, probably mediated via the enhancer elements within the viral long-terminal repeats (LTRs). In another gene therapy trial for chronic granulomatous disease (CGD), nonmalignant amplification of myeloid clones contributed to the efficacy but occurred due to similar spleen focus-forming virus (SFFV) LTR-mediated activation of *MDS1-EVI1*, *PRDM16*, or *SETBP1* genes [29]. In animal model systems, retroviral vector transgenes have additionally been susceptible to a substantial reduction and variegation in expression largely attributable to DNA methylation and histone deacetylation [34-44].

Although avoidance of integrating vector systems is not currently a viable option for SCID-X1 gene therapy, the prospect of insertional mutagenesis can be significantly reduced by minimizing the risk associated with individual integration events. This is theoretically achievable by the use of vector systems with more favorable integration behavior, such as human immunodeficiency virus-1-derived lentiviral vectors [45], the use of self-inactivating LTRs, and improved expression cassette design, including the avoidance of strong promoter-enhancer elements [46, 47] . The development of leukemia as a consequence of vector-mediated genotoxicity in gene therapy trials for X-linked severe combined immunodeficiency (SCID-X1) has prompted substantial research effort into the design and safety testing of integrating vectors. In the study done by Samantha L Ginn *et al,* evaluation of the efficacy and safety of lentiviral vectors containing the promoters from either the elongation factor-1-α (*EF1α*), phosphoglycerate kinase (*PGK*), or Wiscott–Aldrich syndrome (*WAS*) genes to drive expression of the human γc transgene in a murine model of SCID-X1 was carried out [48].

A self-inactivating lentiviral vector expressing the human *IL2RG* cDNA was constructed based on the pRRLsin18.cPPT.WPRE vector backbone. In this construct, *Pac* I restriction endonuclease sites were introduced to flank the γc expression cassette (Fig. 2). Polylinkers containing multiple rare unique sites were also introduced to facilitate sequential "retrofitting" of the construct with additional elements to optimize efficacy and safety in future applications. In this study, the human EF1α, human PGK, and human WAS promoters were selected to drive expression of γc based on their range of transcriptional activities in the ED-7R human T-cell line, with the promoter from the EF1α gene being approximately five- and nine fold more active in this line compared with the promoters from the *PGK* and *WAS* genes, respectively. In addition, a vector construct containing an EF1α-EGFP expression cassette was made and used as a control to transduce C57Bl/6 progenitor cells.

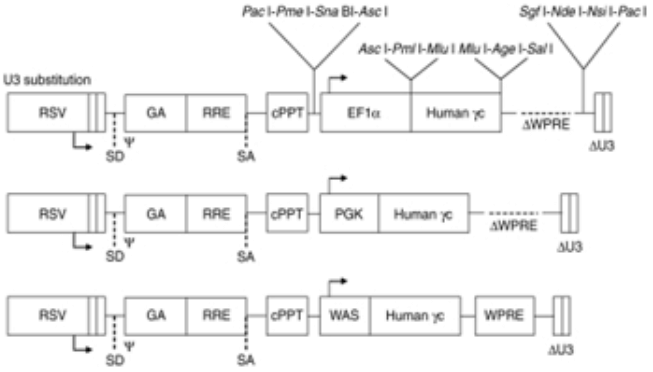

Fig. 2: Lentiviral vector constructs used in this study. Vectors contained the promoter elements from either the 1,177 base-pair (bp) human elongation factor-1-α (EF1α), 516 bp human phosphoglycerate kinase (PGK), or 481 bp human Wiskott–Aldrich syndrome protein (WAS) genes to drive expression of the human γc cDNA. ψ, packaging and dimerization signal; GA, fragment of the HIV-1 gag gene; cPPT, central polypurine tract; RRE, Rev responsive element; RSV, Rous sarcoma virus hybrid promoter; SD/SA, splice-donor and spice-acceptor sites; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. The locations of unique and rare restriction endonuclease sites are indicated.

A restoration of lymphoid development (Fig. 3) and immune function (Fig. 4) in animals treated with progenitor cells transduced by the EF1α-containing vector was observed. Immune reconstitution was poor in mice receiving cells modified with either the PGK or WAS-containing constructs. Long-term follow-up of mice reconstituted with either $IL2RG^{-/-}$ cells treated with the EF1α-γc vector or C57Bl/6 cells treated with EF1α-EGFP revealed a statistically significant incidence of lymphoma in the former group. In all instances, the malignant clones arose from γc-transduced $IL2RG^{-/-}$ progenitor cells, despite an approximately fourfold excess of untransduced $IL2RG^{-/-}$ cells in the reinfused population. Importantly, however, this correlation between receipt of gene therapy and tumor formation could not be attributed to insertional mutagenesis or γc over expression, illustrating the significant limitations of murine models in the assessment of vector safety. Thus, these findings point to the existence of other ill-defined risk factors for oncogenesis, such as transduced progenitors being driven to undergo supraphysiological levels of replication (replicative stress), in

gene therapy protocols targeting the hematopoietic compartment, and highlight the need for detailed mechanistic analysis of tumor readouts observed in preclinical animal models.

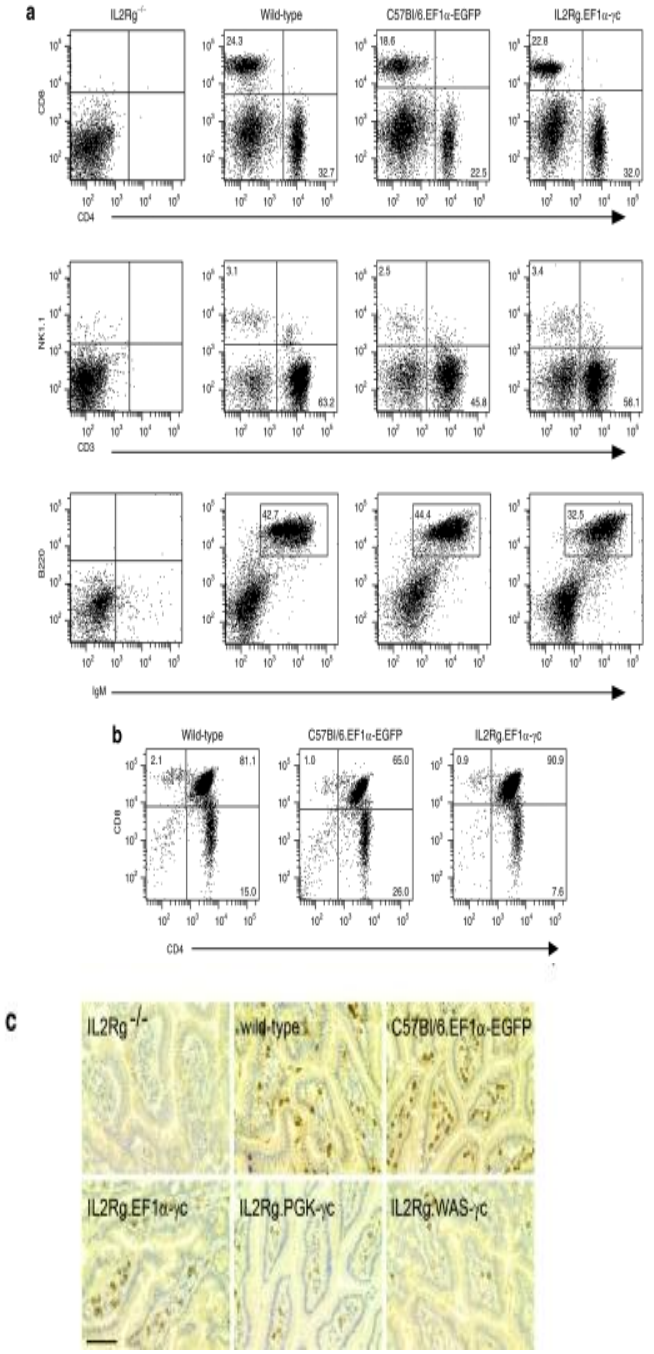

Fig. 3: Restoration of lymphocyte populations following lentiviral vector–mediated gene transfer. (a) Splenocytes from transplant recipients were examined by flow cytometry using antibodies against murine B220, CD3, CD4, CD8, IgM, and NK1.1. (b) Thymopoiesis in transplant recipients receiving vector-treated progenitors was examined by flow cytometry using antibodies against CD3, CD4, and CD8. (c) Small intestinal samples were stained for CD3 and revealed intraepithelial lymphocyte development in wild-type mice and recipient mice following gene therapy. Bar = 100 μm.

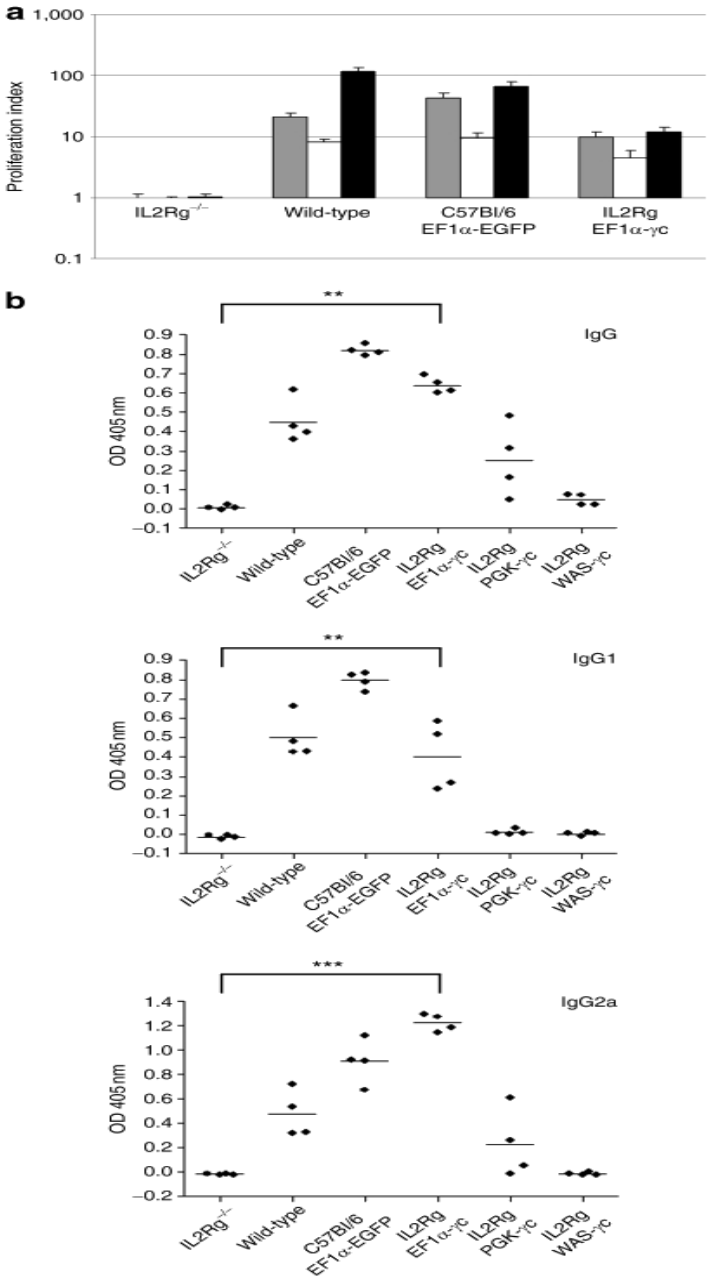

*Fig. 4: Restoration of immune function following lentiviral vector–mediated gene transfer. (a) Splenocytes from transplant recipients or wild-type mice were stimulated under conditions to promote T-cell proliferation. Proliferating cells were evaluated by the incorporation of [3H] thymidine and expressed as the ratio of counts obtained for stimulated to unstimulated cells. Gray bars, conA alone; white bars, IL-2 alone; black bars, conA and IL-2. (b) Humoral immune responses, indicated by serum IgG, IgG1, and IgG2a levels, were examined in mice transplanted with vector-treated C57Bl/6 or IL2RG$^{-/-}$ progenitors, and compared to $\gamma c^{-/-}Rag2^{-/-}c5^{-/-}$ mice transplanted with untransduced IL2RG$^{-/-}$ cells. Histograms represent the mean value for each group (n = 4) with error bars representing the standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.0001 (Wilcoxon rank-sum test). EF1α, elongation factor-1-α. OD, optical density; PGK, phosphoglycerate kinase; WAS, Wiskott–Aldrich syndrome.*

To develop improved strategies for hematopoietic cell gene therapy, Fang Zhang *et al* had assessed the potential of the

novel human *HNRPA2B1-CBX3* UCOE (A2UCOE) within the context of a self-inactivating (SIN) lentiviral vector [49]. Ubiquitously acting chromatin opening elements (UCOEs) consist of methylation-free CpG islands encompassing dual divergently transcribed promoters of housekeeping genes that have been shown to confer resistance to transcriptional silencing and to produce consistent and stable transgene expression in tissue culture systems. Unlike viral promoters, the enhancer-less A2UCOE gave rise to populations of cells that expressed a reporter transgene at a highly reproducible level. The efficiency of expression per vector genome was also markedly increased in vivo compared with vectors incorporating either spleen focus-forming virus (SFFV) or cytomegalovirus (CMV) promoters, suggesting a relative resistance to silencing. Furthermore, an A2UCOE-*IL2RG* vector fully restored the IL-2 signaling pathway within IL2RG-deficient human cells in vitro and successfully rescued the X-linked severe combined immunodeficiency (SCID-X1) phenotype in a mouse model of this disease. These data indicate that the A2UCOE displays highly reliable transcriptional activity within a lentiviral vector, largely overcoming insertion-site position effects and giving rise to therapeutically relevant levels of gene expression. These properties are achieved in the absence of classic enhancer activity and therefore may confer a high safety profile.

5. FANCONI ANEMIA

Fanconi anemia (FA) is a hereditary syndrome characterized by bone marrow failure and a predisposition to cancer. While mutations that cause the disease have been identified in 13 genes, mutations of *FANCA* are the most common molecular etiology of FA in humans [50, 51]. The cumulative risk of bone marrow failure by age 40 in FA patients is 90%, with a median age of onset of 8 years [52]. FA patients are also prone to develop leukemia and cancer, particularly acute myelogenous leukemia and squamous cell carcinoma [52, 53]. Currently, allogeneic stem-cell transplantation is the only curative treatment for bone marrow failure.[17] Ten-year survival following matched sibling transplantation is >80% [54]. However, unaffected matched sibling donors are unavailable to the majority of the patients, and the results of alternative donor transplantation remain unsatisfactory [55].

Genetic correction of autologous hematopoietic stem cells (HSCs) provides a potential alternative for patients lacking a matched related donor. γ-Retroviral and lentiviral vectors have been employed successfully to deliver complementing FA-cDNA to HSCs derived from mice with targeted disruptions of the *Fanca* and *Fancc* genes[56-59]. Two earlier clinical gene therapy trials employing γ-retroviral vectors have provided proof-of-principle for the effectiveness of these vector agents in correcting the cellular FA phenotype in primary human FA $CD34^+$ cells [60, 61]. However, these trials failed to achieve significant levels of engraftment of transduced cells and provided limited, if any, clinical benefit.

Taking into account that genetically corrected FA hematopoietic stem cells either by natural processes [62, 63] or after *ex vivo* gene therapy [64] acquire a proliferation advantage *in vivo*, major aspects should account for the limited efficacy of gene therapy protocols already used in FA patients.

In this respect, either the reduced number of transplanted $CD34^+$ cells or qualitative HSC defects associated to a disruption in the FA pathway [65, 66] might account for the difficulties to engraft FA patients with corrected cells [61, 67, 68]. Additional aspects need, however, to be considered aiming to improve the efficacy of gene therapy in FA. First, although the repopulation potential of purified $CD34^+$ cells from X-linked severe combined immunodeficiency, adenosine deaminase deficient severe combined immunodeficiency, and chronic granulomatous disease patients is evident [69-72], the question of whether purified $CD34^+$ cells from FA patients will be capable of engrafting these patients needs to be considered with caution, because FA is an HSC disease where the reproducible expression of the CD34 antigen in FA HSCs has not been demonstrated.

The most recent gene therapy clinical trial in *FANCA* patients utilized retrovirus-mediated gene transfer [68]. Unfortunately, successful, durable engraftment of gene-modified cells was not achieved. The attempts were also thwarted by inability to adequately mobilize hematopoietic stem cells in these patients with bone marrow failure. Critical to the future success of gene therapy in FA patients is the development of a rapid transduction (RT) protocol that will minimize the time in culture for fragile FA stem cells, achieve high transduction efficiency, ensure therapeutic expression levels of the transgene, and preserve the engraftment capability. Lentiviral vectors provide an advantage in that there can be shorter transduction time, minimizing ex vivo culture, and high level transgene expression in hematopoietic cells. A study of lentiviral transduction of murine $Fanca^{-/-}$ hematopoietic stem cells demonstrated the ability to successfully transduce with an MOI of 100, with preservation of engraftment capability and phenotypic correction [73] Another group reported successful in vitro correction of FA patient cells with lentiviral transduction and improved recovery of $CD34^+$ cells using hydroxyethyl starch isolation of white blood cells [74]. Thus, lentiviral transduction appears to be a promising advance.

In a study using rapid lentiviral transduction method, Lars UW Muller aimed to preserve the engraftment potential of $Fanca^{-/-}$ HSCs [73]. The lentiviral vector used consisted of a self-inactivating lentiviral backbone with an internal spleen

focus-forming virus promoter and a truncated woodchuck hepatitis virus post-transcriptional regulatory element, devoid of X-protein coding sequences. A panel of expression vectors was generated, including (i) a vector expressing the enhanced GFP gene (referred to as GFP vector), (ii) the MGMT $MGMT^{P140K}$ and GFP genes (MG vector), and (iii) a tricistronic vector containing the *Fanca*, $MGMT^{P140K}$, and GFP genes (FMG vector) (Fig. 5).

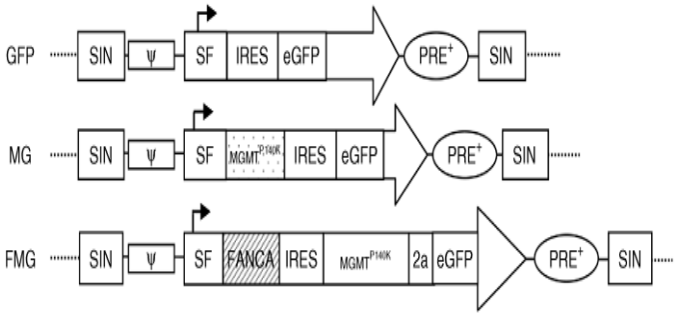

Fig. 5: Schematic representation of the lentiviral vectors used in this study. Vectors are referred to as (i) GFP vector (mono-cistronic GFP-expressing construct), (ii) MG-vector (bi-cistronic $MGMT^{P140K}$/GFP construct), and (iii) FMG (tricistronic FANCA/$MGMT^{P140K}$/GFP construct). SIN, self-inactivating deletion of the viral U3 promoter; Ψ, packaging signal, SF, Spleen focus-forming virus U3 promoter; IRES, internal ribosome entry site of the encephalomyocarditis virus; eGFP, enhanced green fluorescent protein; $MGMT^{P140K}$, P140K mutant of O^6-methylguanine-DNA-methyltransferase; 2A, self-cleaving esterase from foot-and-mouth disease virus; PRE*, woodchuck hepatitis post-transcriptional regulatory element, modified, devoid of X-protein coding sequences.

These LVs were utilized to transduce Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) bone marrow cells isolated from $CD45.2^+$ $Fanca^{-/-}$ or wild-type (wt) mice. These transplantation models demonstrated a profound engraftment defect in $Fanca^{-/-}$ HSCs under the stress of conventional prestimulation/conventional transduction (CT) conditions. After either CT or RT, the progeny cells derived from 2,000 LSK cells were injected into lethally irradiated $CD45.1^+$ wild type (wt) mice. Observation endpoints were engraftment, transduction efficiency, and donor chimerism at 4 months after transplantation. They observed a striking difference in the rate of engraftment failure between the two transduction methods. Engraftment failure was defined by the presence of severe pancytopenia in moribund mice during days 10–21 after transplantation. Engraftment failure was specific to $Fanca^{-/-}$ HSCs and was not observed in the wt setting, thereby emphasizing the differences in HSC vigor between $Fanca^{-/-}$ and wt HSCs under these conditions. RT preserved the engraftment of $Fanca^{-/-}$ HSCs to the wt levels. The engraftment of gene-modified cells was similarly affected. Despite comparable *in vitro* transduction rates between the RT and CT approaches, the total engraftment of the transduced cells was superior in recipients of RT HSCs. Importantly, rapid lentiviral transduction most likely occurred with respect to HSCs, as evidenced by the GFP marking seen in all the hematopoietic lineages in primary and secondary transplant recipients. RT was performed with a significant excess of viral particles (multiplicity of infection of 100), however, transduction rates were modest overall, ranging between 1 and 10%. A detailed analysis was carried out of the number of proviral copies in gene-marked green fluorescent protein (GFP^+) progenitor colonies derived from bone marrow transplant recipients, and the results of the analysis highlight one potential weakness of this approach. An average of three to four vector copies/cell were observed after RT, thereby suggesting that not all stem cells contained in the LSK fraction were equally susceptible to lentiviral transduction during the 4-hour transduction period. While there is some emerging evidence that lentiviral transduction may pose less risk for insertional mutagenesis [75,76], additional adjustment of the transduction conditions with the goal of transducing more stem cells with fewer copies per cell were clearly indicated. Further study of this approach of rapid lentiviral transduction of FA HSCs [73, 77] in the context of human HSCs is warranted in order to facilitate translation into a clinical trial.

One of the major hurdles for the development of gene therapy for Fanconi anemia (FA) is the increased sensitivity of FA stem cells to free radical-induced DNA damage during ex vivo culture and manipulation. To minimize this damage, Becker et al (2010) developed a brief transduction procedure for lentivirus vector-mediated transduction of hematopoietic progenitor cells from patients with Fanconi anemia complementation group A (*FANCA*). The lentiviral vector FancA-sW contained the phosphoglycerate kinase promoter, the *FANCA* cDNA, and a synthetic, safety-modified woodchuck post transcriptional regulatory element (sW). Bone marrow mononuclear cells or purified $CD34^+$ cells from patients with *FANCA* were transduced in an overnight culture on recombinant fibronectin peptide CH-296, in low (5%) oxygen, with the reducing agent, N-acetyl-L-cysteine (NAC), and a combination of growth factors, granulocyte colony-stimulating factor (G-CSF), Flt3 ligand, stem cell factor (SCF), and thrombopoietin. Transduced cells plated in methylcellulose in hypoxia with NAC exhibited increased colony formation compared to 21% oxygen without NAC ($P < 0.03$), demonstrated increased resistance to mitomycin C compared to green fluorescent protein (GFP)-transduced controls ($P < 0.007$), and increased survival. Thus, this study showed that combining short transduction and reducing oxidative stress may enhance the viability and engraftment of gene-corrected cells in patients with *FANCA [78].*

6. LYSOSOMAL STORAGE DISEASE- FARBER DISEASE AND FABRY DISEASE

Lysosomal storage disorders (LSDs) are a group of over 40 distinct metabolic conditions resulting from deficient activity of enzymes involved in macromolecule digestion. While

individually their prevalence is low, as a group they can occur at high frequencies (up to 1 in 7,700) in some populations [79]

6.1. Farber Disease

Farber disease is a rare, autosomally inherited LSD caused by mutation of the gene (ASAH1) encoding N-acylsphingosine deacylase (acid ceramidase, AC; EC 3.5.1.23), a protein that catabolizes the hydrolysis of ceramide into sphingosine and free fatty acids [80, 81] While the phenotype of the disease varies, most patients present with a characteristic triad of symptoms: subcutaneous granulomas, a hoarse cry, and painful swollen joints[80] In the classic and most severe type of Farber disease, patients also show progressive neurological deterioration and patients typically die by the age of two [80]. Treatment consists primarily of palliative care such as corticosteroids for the pain, tracheostomy to relieve respiratory difficulties, and surgery to remove the granulomas [81, 82].Allogeneic bone marrow transplantation (BMT) has been attempted for some Farber patients based on the reasoning that a population of cells with normal enzyme activity could ameliorate the effects of the deficient enzyme[83,84]. Farber disease is an attractive target for gene therapy since it is caused by a single gene defect, and the cDNA of AC has been cloned. In addition, the enzyme is well-characterized. There are a number of approaches that have been used to introduce therapeutic genes in vivo. Viral methods using vectors such as retroviruses offer the advantage of long-term gene transfer since the viral DNA can integrate into the host genome and can be transmitted to progeny cells. Transduction of bone marrow-derived cells with a viral vector engineered to over express a therapeutic transgene can potentially provide a systemic, circulating source of enzyme [84].

In a study done by Shobha Ramsubir *et al,* a novel retroviral (RV) vectors and lentiviral (LV) vectors that engineer co-expression of AC and a cell surface marking transgene product, human CD25 (huCD25) was employed [84]. As a complete deletion of the AC gene is embryonic lethal and the heterozygous animals are not widely available, surrogate models for *in vivo* testing of gene therapy strategies for the treatment of Farber disease was developed. To confirm that the viral vectors constructed could infect cells and produce functional enzyme, transduction of Farber patient B cells and fibroblasts was carried out. High levels of huCD25 expression from transduced cells were detected and were able to use the huCD25 marker to enrich the pool of transduced B cells. Measurement of AC activity showed that transduced cells had significantly increased enzyme activity and decreased ceramide storage following transduction. They also showed that functional enzyme was secreted and could be taken up and utilized by non-transduced cells. The transduction of HSPCs is an attractive option for treating a number of LSDs since these cells can provide a circulating source of the therapeutic factor. In order to test the effect of transduction on the engraftment of human cells, a NOD/SCID xenotransplantation model that is commonly employed to assess human cell engraftment was used. Transduction of $CD34^+$ (a prime target for treatment of patients with Farber disease) cells with LV/AC/huCD25 was carried out and these were transplanted into irradiated NOD/SCID mice. High levels of human cell engraftment as assessed by measurement of CD45 expression was achieved and found that engrafted cells expressed the huCD25 marking gene in the bone marrow. The effect of delivering an LV expressing AC and huCD25 directly to neonatal animals was also studied. In this aspect of the study, a normal neonatal mouse was injected with LV/AC/huCD25 and found that up to 14 weeks post-injection, sCD25 was detected in the plasma, suggesting the persistence of vector and long-term transgene expression. Non-treated mice did not have any detectable levels of sCD25 (data not shown). Therapeutic transgene expression was further evidenced by the increased AC activity found in the livers of mice treated with LV/AC/huCD25 as compared to wild-type mice.

The results of these studies represented significant progress towards the development of gene therapy strategies for Farber disease. However, this approach is limited by the requirement of early diagnosis e.g., after prenatal diagnosis or, when available, newborn screening for LSDs.

6.2. Fabry Disease

Fabry disease is a lysosomal storage disorder (LSD) that is due to an inherited deficiency of the lysosomal enzyme -galactosidase A (-gal A; EC 3.2.1.22) [85]. The disease is characterized by the systemic accumulation of galactosylsphingolipids, mainly globotriaosylceramide (Gb3) [86]. Fabry disease has a prevalence of 1/40 000 males and is very heterogenous at the molecular level, with more than 245 mutations reported [87]. It also shows variability at the clinical level, with some patients having a variant or attenuated form of the disease [88]. Although enzyme replacement therapy (ERT) is currently available for Fabry disease with reports of improvement in some clinical and pathological manifestations [89], sustained and frequent administration of the factor is required. Alternative treatment is by using allogeneic stem cell transplantation (SCT) has been reported [90]. In general, outcomes have been long-term but only partly effective. There are two major reasons limiting the clinical application of allogeneic SCT. First, there is a limited availability of human lymphocyte antigen-matched donors and a significant risk of treatment-related morbidity or mortality owing to severe graft-versus-host disease (GvHD) [91]. Secondly, non-modified hematopoietic stem/progenitor cells (HSPCs) and their progeny do not secrete therapeutic levels of target enzyme, which can be taken up at a distance by affected organs in a

process termed 'metabolic cooperativity' or 'cross-correction'. Autologous SCT alleviates risks involved with GvHD and will likely be even more effective at systemic correction of LSDs and other disorders if the target cells have been genetically modified to over-produce and secrete the therapeutic enzyme before transplantation. Virus-based delivery of genes can correct cells and establish a sustained supply of therapeutic proteins.

In a study, M Yoshimitsu *et al* demonstrated efficient and long-term LV-mediated gene transfer into syngeneic HSPCs of Fabry mice with a single dose of concentrated viral supernatant [92]. A recombinant LV that engineered expression of α-gal A (LV/α-gal A) in a monocistronic format was employed without a cell surface marker that could have been used for enrichment of transduced cells. The therapeutic levels of transgene expression in the study using the monocistronic vector construct facilitates practical application of this approach in the clinic without possible confounding effects due to coexpression of a marking transgene. A downside of this, however, is that accurate titering of functional vector stocks and long-term detection of vector in transplanted recipients is more complicated. In this study, transplanted Fabry mice with bone marrow mononuclear cells (BMMNCs) transduced a single time with a LV encoding the human α-gal A cDNA. The in vitro and in vivo studies showed that transduced cells not only expressed the functional therapeutic transgene product intracellularly but also secreted the corrective factor at appreciable levels. LV/α-gal A-treated mice showed supranormal α-gal A activity in plasma over 24 weeks post BMT. Plasma α-gal A activity from treated Fabry mice was two-fold higher than wild-type controls. In addition, as expected, α-gal A activity in the therapeutically transduced recipients was significantly higher in all organs assessed. These data also suggest that correction of only relatively a small number of key cells, such as repopulating HSPCs, may be sufficient to cure Fabry disease. Importantly, Fabry disease is also an appropriate gene therapy target in this regard, as over expression of α-gal A to high levels in various organs (up to 11000-fold over background) in transgenic mice is well tolerated. Colony PCR assays and then a secondary BMT was also performed to confirm LV-mediated transduction of more primitive hematopoietic cells and also looked for an extended corrective effect. Twenty weeks after the secondary transplantation, a sustained and clinically relevant level of α-gal A activity in plasma and in multiple organs were observed. Lastly, transduction of mobilized PB $CD34^+$ cells from a Fabry patient was carried out and corresponding enzymatic increases was observed.

These results indicated that transplantation of single-time LV-transduced autologous HSPCs may represent a long-sought therapeutic option for Fabry disease patients and possibly for other LSDs.

7. CONCLUSION

Lentiviral vectors have now become commonplace in experimental research. Despite the progress made in LV technology and the demonstration in multiple preclinical studies that animal models suffering from the cognate human disease can effectively be treated by LV-based gene therapy, there are still significant challenges ahead. In particular, the performance of LVs in patients may not necessarily mimic what has been observed in mouse models. Therefore, there is a need to conduct preclinical studies in large animal models to help bridge the gap between early proof-of-concept preclinical studies and clinical trials. The lack of pre-existing immunity to vector components in most subjects may give LVs a possible advantage over other vector systems derived from viruses that are more widespread in the human population, such as AAV or adenovirus. However, it will be critically important to assess the adaptive and innate immune responses following LV-based gene therapy in subjects enrolled in clinical trials, particularly because the immune system poses a significant challenge for *in vivo* gene therapy with other viral vector systems. Though the safety profile of LVs has significantly improved over that of γ-RVs, integration can still potentially result in oncogene activation and/or inactivation of tumor suppressor genes. Follow-up studies will be needed to assess the long-term safety and stability of LV-mediated gene therapy in patients. Nevertheless, the consequences of LV-mediated gene transfer are expanding rapidly and the technology is evolving providing an attractive alternative to other vector systems.

8. REFERENCES

1. Grinshpun A, Condiotti R, t al. *Mol Ther*. 2010 September; **18(9):** 1592–1598.
2. Sinn PL, Arias AC, et al. *J Virol*. 2008; 82:10684–10692.
3. Chou JY. *Curr Mol Med.* 2001; **1:**25–44.
4. Yiu, W H, Lee Y M, et al. *Mol Ther*. 2010 June; **18(6):** 1076–1084.
5. Salganik SV, Weinstein DA, et al. *Lab Invest*. 2009; **89:**1032–1042.
6. Koeberl DD, Pinto C, et al. *Mol Ther*. 2008; **16:**665–672.
7. Koeberl DD, Sun B, et al. *Mol Ther*. 2007; **15:**1253–1258.
8. Lucarelli G, et al. *Blood Rev*. 2002; **16:**81–85.
9. Miccio A, Cesari R, et al. *Proc Natl Acad Sci USA*. 2008; **105:**10547–10552.
10. Zhao H, Pestina T I, et al. *Blood*. 2009; **113(23):** 5747–5756.
11. Cai S, Ernstberger A, et al. *Exp Hematol*. 2008; **36:**283–292.
12. May C, et al. *Nature*. 2000; **406:**82–86.
13. Imren S, et al. *Proc Natl Acad Sci USA*. 2002; **99:**14380–14385.
14. Puthenveetil G, et al. *Blood*. 2004; **104:**3445–3453.
15. Hargrove P, Kepes S, et al. *Mol Ther.* 2008; **16:**525–533.
16. Sugamura K, Asao Het al. *Adv Immunol.* 1995; **59**:225–277.
17. Asao H, Okuyama C, et al. *J Immunol.* 2001;**167**:1–5

18. Noguchi M, Nakamura Yet al. *Science.* 1993; **262**:1877–1880.
19. Buckley RH, Schiff RI, et al. *J Pediatr.* 1997; **130**:378–387.
20. Cavazzana-Calvo M, et al. *Blood.* 1996; **88**:3901–3909.
21. Puel A, Ziegler SF,et al. *Nat Genet.* 1998; **20**:394–397.
22. Buckley RH, Schiff SE, et al. *N Engl J Med.* 1999; **340**:508–516.
23. Antoine C, Müller et al. *Lancet.* 2003; **361**:553–560.
24. Cavazzana-Calvo M, et al. *Science.* 2000; **288:**669–672.
25. Aiuti A, Slavin S, Aker M, et al. *Science*. 2002; **296**:2410–2413.
26. Gaspar HB, Parsley KL, et al. Lancet. 2004; **364:**2181–2187.
27. Gaspar HB, Bjorkegren E, et al. *Mol Ther.* 2006; **14:**505–513.
28. Schmidt M, et al. *Blood.* 2005; **105:**2699–2706.
29. Ott MG, Schmidt M, et al. *Nat Med*. 2006; **12:**401–409.
30. Hacein-Bey-Abina S, et al. *Science*. 2003; **302:**415–419.
31. Hacein-Bey-Abina S, et al. *N Engl J Med*. 2003; **348:**255–256.
32. Seggewiss R, et al. *Blood.* 2006; **107:**3865–3867.
33. Nienhuis AW, et al. *Mol Ther*. 2006; **13:**1031–1049.
34. Hoeben RC, et al. *J Virol*. 1991; **65:**904–912.
35. Challita PM, Kohn DB. *Proc Natl Acad Sci* U S A. 1994; **91:**2567–2571.
36. Razin A. *EMBO J.* 1998; **17:**4905–4908.
37. Klug CA, Cheshier S, Weissman IL. *Blood.* 2000; **96:**894–901.
38. Lorincz MC, et al. *Mol Cell Biol.* 2002; **22:**7572–7580.
39. Yao S, et al. *Nucleic Acids Res*. 2003; **31:**5317–5323.
40. Ellis J. *Hum Gene Ther*. 2005; **16:**1241–1246.
41. Sinn PL, et al. *Gene Ther*. 2005; **12:**1089–1098.
42. Doerfler W, et al. *Dev Biol (*Basel) 2001; **106:**89–97.
43. Rosenqvist N, et al. *J Gene Med*. 2002; **4:**248–257.
44. Williams S, Mustoe T, et al. *BMC Biotechnol*. 2005; **5:**17.
45. De Palma M, Montini E et al. *Blood.* 2005; **105**:2307–2315.
46. Thornhill SI, et al. *Mol Ther.* 2008; **16:**590–598.
47. Montini E, et al. *J Clin Invest.* 2009; **119**:964–975.
48. Ginn S, Liao S, et al. *Mol Ther.* 2010 May; **18(5):** 965–976.
49. Fang Zhang, Susannah I. et al. *Blood*. 2007 September 1; **110(5):** 1448–1457.
50. Taniguchi, T and D'Andrea, AD. *Blood,* 2006; **107**: 4223–4233.
51. Smogorzewska, A, *et al*. *Cell,* 2007; **129**: 289–301.
52. Kutler, DI, *et al*. *Blood,* 2003; **101**: 1249–1256.
53. Rosenberg, PS, et al. *Blood,* 2005; **105**: 67–73.
54. Farzin, A, *et al*. *Br J Haematol,* 2007; **136**: 633–640.
55. Wagner, JE, Eapen, M, MacMillan, ML, Harris, RE, Pasquini, R, Boulad, F *et al*. *Blood,* 2007; **109**: 2256–2262
56. Liu, JM, Young, *et al*. *Hum Gene Ther,* 1997; **8**: 1715–1730.
57. Yamada, K, *et al*. *Mol Ther,* 2003; **8**: 600–610.
58. Galimi, F, Noll, *et al*. *Blood,* 2002; **100**: 2732–2736.
59. Rio, P, *et al*. *Blood,* 2002; **100**: 2032–2039.
60. Kelly PF, *et al*. *Mol Ther,* 2007; **15**: 211–219.
61. Liu, JM, Kim, S, *et al*. *Hum Gene Ther,* 1999; **10**: 2337–2346.
62. Waisfisz Q, et al. *Nat Genet.* 1999; **22:**379–383.
63. Gregory JJ, et al. *Proc Natl Acad Sci USA*. 2001; **98:**2532–2537.
64. Rio P, et al. Blood. 2008; **112:**4853–4861.
65. Carreau M, et al. *Exp Hematol*. 1999; **27:**1667–1674.
66. Rio P, Segovia JC, et al. *Blood.* 2002; **100:**2032–2039.
67. Walsh CE, Fu K, et al. *Blood*. 2001; **98:**718a.
68. Kelly PF, Radtke S, et al. *Mol Ther*. 2007; **15:**211–219
69. Cavazzana-Calvo M, et al. *Science.* 2000; **288:**669–672.
70. Gaspar HB, Parsley KL, et al. *Lancet*. 2004; **364:**2181–2187.
71. Aiuti A, et al. *Science.* 2002; **296:**2410–2413.
72. Ott MG, Schmidt M, et al. *Nat Med*. 2006; **12:**401–4
73. Müller LU, et al. *Mol Ther*. 2008; **16:**1154–1160.
74. Jacome A, et al. *Mol Ther*. 2009; **17:**1083–1092.
75. Elena Almarza, Paula Río, et al. *Molecular Therapy,* 2007; **158:** 1487–1494.
76. Ángel Raya, et al. *Nature.* 2009; **460(7251):** 53–59.
77. Ariana Jacome, et al. *Mol Ther.* 2009; **17(6):** 1083–1092.
78. Pamela S. Becker, et al. *Gene The*r. 2010; **17(10):** 1244–1252.
79. Meikle PJ,et al. *JAMA*. 1999; **281:**249–254.
80. Moser HW. Acid Ceramidase Deficiency: Farber Lipogranulomatosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill; 2001. pp. 3573–3588.
81. Bar J, et al. *Hum. Mutat*. 2001; **17:**199–209.
82. Haraoka G, et al. *Ann. Plast. Surg.* 1997; **39:**405–410.
83. Yeager AM,et al. *Bone Marrow Transplant*. 2000; **26:**357–363.
84. Shobha Ramsubir, Takahiro Nonaka, et al. *Mol Genet Metab*. 2008 November; **95(3):** 133–141.
85. Brady RO et al. *N Engl J Med* 1967; **276:** 1163–1167.
86. Desnick RJ, Ioannou YA, Eng CM. Fabry disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill Inc.: New York, 2001, pp 3733–3774.
87. Garman SC, Garboczi DN. *J Mol Biol* 2004; **337:** 319–335.
88. Nakao S, *et al*. *N Engl J Med* 1995; **333:** 288–293.
89. Schiffmann R, *et al*. *JAMA* 2001; **285:** 2743–2749.
90. Staba SL, *et al*. *N Engl J Med* 2004; **350:** 1960–1969.
91. Krivit W, et al. *Curr Opin Neurol* 1999; **12:** 167–176.
92. M Yoshimitsu, et al. *Gene Therapy*, 2007; **14:** 256–265.