Stem Cell Gene Therapy for Fanconi Anemia: Report from the 1st International Fanconi Anemia Gene Therapy Working Group Meeting

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Survival rates after allogeneic hematopoietic cell transplantation (HCT) for Fanconi anemia (FA) have increased dramatically since 2000. However, the use of autologous stem cell gene therapy, whereby the patient's own blood stem cells are modified to express the wild-type gene product, could potentially avoid the early and late complications of allogeneic HCT. Over the last decades, gene therapy has experienced a high degree of optimism interrupted by periods of diminished expectation. Optimism stems from recent examples of successful gene correction in several congenital immunodeficiencies, whereas diminished expectations come from the realization that gene therapy will not be free of side effects. The goal of the 1st International Fanconi Anemia Gene Therapy Working Group Meeting was to determine the optimal strategy for moving stem cell gene therapy into clinical trials for individuals with FA. To this end, key investigators examined vector design, transduction method, criteria for large-scale clinical-grade vector manufacture, hematopoietic cell preparation, and eligibility criteria for FA patients most likely to benefit. The report summarizes the roadmap for the development of gene therapy for FA.

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"*…if you wait for the robins, spring will be over.*"
—Warren Buffett

INTRODUCTION

The goal of gene therapy in Fanconi anemia (FA)[1] is to develop a safe treatment for bone marrow (BM) failure, while preventing leukemia and reducing the risk for subsequent mucocutaneous squamous cell carcinomas. The potential benefit of hematopoietic cell gene therapy, even if a relatively low frequency of hematopoietic stem/progenitor cells (HSC/HPCs) express the wild-type gene product, is supported by the observations that some individuals with FA develop self-correcting mutations in one of the FA genes, resulting in a single corrected stem cell that is capable of repopulating the BM and leading to normal hematopoiesis.[2–7] The magnitude of the compensatory genetic changes in FA mosaicism is, however, unpredictable both in the gene location and cell type.

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Thus, gene addition using viral vectors has emerged as a leading technology to harness this concept in a clinically meaningful way.

The first meeting of an International Fanconi Anemia Gene Therapy Working Group, held on 16 November 2010, was organized and funded by the Fanconi Hope Charitable Trust, UK, and the Fanconi Anemia Research Fund, OR, USA. This meeting, hosted by Adrian Thrasher at the Institute of Child Health and chaired by Jakub Tolar from the University of Minnesota, brought together leading gene therapy and FA experts from across the globe to create an action plan for gene therapy trials in FA.

The need to run FA trials collaboratively stems from the very rare nature of the condition; it is estimated that the frequency is 1 in 350,000 births. To date, 14 causative genes have been identified, although the molecular pathology in ~60% of FA patients is a consequence of the loss-of-function mutation in one of them, *FANCA*.[8] Due to inefficient DNA repair, particularly in cell division-associated homologous recombination, and dysregulation of signaling pathways guiding cell proliferation and apoptosis, individuals with FA typically develop congenital defects (*e.g.*, growth retardation and radial ray defects), severe hematopoietic defects (*e.g.*, aplastic anemia), and cancer.[9,10] Hematopoietic cell transplantation (HCT) has been the only curative measure for BM failure and leukemia in FA to date.[11] Outcomes of HCT have been optimized by offering HCT at an earlier age, using fludarabine-based conditioning regimens, and employing graft T-cell depletion. In recent studies, recipients of FA-negative, human leukocyte antigen (HLA)-identical, sibling grafts had 3-year overall survival after HCT of 69–93%; using alternative donor grafts, survival was 52–88%.[11–14] Even though HCT is a life-saving procedure for the majority of people with FA, intensive pre-HCT conditioning of the host with chemotherapy or chemoradiotherapy is needed both to make space for the new hematopoietic system and to reduce the likelihood of rejection of the allogeneic donor hematopoietic graft by the host's immune system. The life-threatening complications include sinusoidal obstructive syndrome, graft failure, and infections. Moreover, multiple drugs, each with their own set of side effects, have to be used to prevent graft-versus-host disease (GVHD, the donor antihost immunological complication of allogeneic HCT), which is a multi-organ-system destructive disorder due to donor T-cell immune recognition of minor and/or major histocompatibility antigens in the host. Incidence of severe acute GVHD in recipients of matched-sibling donor grafts is typically <10%.[11,13,15] Patients receiving unrelated donor grafts (even with T-cell depletion) had an incidence of acute GVHD requiring additional immunosuppressive therapy in 12.5–34%.[11,12,16–18] Of note, it is possible that GVHD and the genotoxic agents used as a conditioning regimen may increase the risk of subsequent FA-specific malignancies.[19,20] Each of these complications and their treatments predispose the recipient to bacterial, fungal, and viral infections that are themselves a significant source of morbidity and mortality after allogeneic HCT. Importantly, most of these complications could, in principle, be diminished (*e.g.*, infections and sinusoidal obstructive syndrome) or avoided (*e.g.*, GVHD) with autologous HCT with patient-specific, gene-corrected stem cells.

The basis of viral-mediated gene correction is the permanent insertion of the therapeutic gene in the host genome. As an undesired side effect, however, several patients treated for X-linked severe combined immunodeficiency or chronic granulomatous disease developed life-threatening hematopoietic cancers driven by the insufficiently controlled, viral-mediated proto-oncogene activation by the vectors that were instrumental in the correction of the immune deficiencies in the first place.[21]

Thus the efficacy and safety of gene therapy is technology-dependent. In the case of FA, the merit of gene therapy will be measured against the natural history of the disease and against the known toxicity of allogeneic HCT, the current treatment of choice for FA. The following summarizes the consensus of this workshop.

VECTOR

Based on broad experience in monogenic disorders in general[21] and limited experience in FA in particular,[22,23] the best-suited vector system for an initial phase 1 clinical trial of gene correction is an HIV-1-derived, self-inactivating lentiviral vector.[24] Similar vectors are currently being tested in a few inherited human disorders. Expression of the human *FANCA* cDNA regulated by an internal human phosphoglycerate kinase (*PGK*) promoter in combination with an optimized woodchuck hepatitis virus posttranscriptional regulatory element (*WPRE*) is likely sufficient for long-term correction of *FANCA* deficient cells[27,28] (**Figure 1**). A recent study has investigated the level of lentiviral-mediated expression of *FANCA* that was necessary to correct the phenotype of human FANCA cells.[29] This study showed that all tested promoters (including the very weak *vav* promoter, the moderately strong *PGK* and cytomegalovirus, *CMV* promoters, and the very potent spleen focus-forming virus, *SFFV* promoter) were equally efficient in correcting the FANCA cellular phenotype, as evidenced by normalization of FANCD2 monoubiquitination, formation of FANCD2 foci, and sensitivity to mitomycin C. Because *PGK*-based lentiviral vectors provide stable transgene expression[30] and low genotoxicity in experimental models[31,32] and in humans,[33] both FANCA gene therapy studies proposed will employ lentiviral vectors where the expression of *FANCA* is regulated by an internal human *PGK* promoter and stabilized by an optimized *WPRE*.[27,29] Transduction of human FA cells is facilitated by inclusion of the vesicular stomatitis virus (VSV-G) envelope.[27] Despite future alternatives for viral envelopes,

Figure 1 The lentiviral vector pC/RRL-PGK-*FANCA*-WPRE. CMV, cytomegalovirus; cPPT, central polypurine tract; *FANCA*, Fanconi anemia cDNA; PGK, phosphoglycerate kinase; RRE, *rev* responsive element; SA, splice acceptor; SD, splice donor; WPRE, safety-optimized version of the woodchuck hepatitis virus posttranscriptional regulatory element.

Table 1 Advantages and limitations of envelopes with a potential application for the gene therapy of Fanconi anemia cells with lentiviral vectors

Envelope	Advantages	Limitations
VSV-G LVs[a]	GMP is well established. High titers of purified LVs are routinely obtained. Already used in clinical trials.[25,26] Transduction of hFA hematopoietic progenitors has been shown.[27]	Rather inefficient for CD34$^+$ cells transduction (high MOIs are generally required). Expensive production. Potential fusogenic effects.
GALV-TR/TM LVs	Efficient transduction of FA progenitor cells at low MOIs.[28] GALV-RVs efficiently transduced hHSCs in clinical trials.[66] Very low toxicity.	GMP production is not established. Not testable in murine models.
RD114-TR LVs	RD114-TR-LVs efficiently transduced human hematopoietic repopulating cells.[35,36] Very stable against human serum. Very low toxicity.	GMP production is not established. Not testable in murine models.
Modified PFV LVs	High titers of purified LVs can be obtained. PFV-LVs efficiently transduced human hematopoietic repopulating cells.[34] Transduction of murine FA stem cells has been shown.[34]	GMP production is not established.
Cocal gp LVs	High titers of purified LVs can be obtained. Cocal gp LVs efficiently transduced human hematopoietic repopulating cells.[37]	GMP production is not established.
EACD LVs	Selective transduction of hCD34$^+$ cells.[38]	GMP production is not established.

Abbreviations: Cocal gp, cocal virus glycoprotein; EACD LVs, LVs displaying early acting cytokines; EACD, early acting, cytokine displaying; GALV, gibbon-ape leukemia virus; GALV-TR/TM LVs, LVs pseudotyped with the GALV-TR or GALV-TM envelope; GMP, good manufacturing practice; hFA, human Fanconi anemia; hHSC, human hematopoietic stem cell; LVs, lentiviral vectors; Modified PFV-LVs, LVs pseudotyped with the foamy virus envelope; MOI, multiplicity of infection; PFV, primate foamy virus; RD114, cat endogenous retroviral envelope gp; RD114-TR, cytoplasmic mutant of RD114; RD114-TR LVs, LVs pseudotyped with RD114; TM, trans-membrane; TR, glycoproteins fused to cytoplasmic tail; VSV-G LVs, LVs pseudotyped with the VSV-G protein; VSV-G, vesicular stomatitis virus.

such as a chimeric gibbon-ape leukemia virus (GALV-TR) envelope[29] or a modified prototype foamy virus envelope[34] and other envelopes,[35–38] (**Table 1**) the participants of the meeting agreed that a VSV-G pseudotyped, self-inactivating lentiviral vector with a *PGK-FANCA-WPRE* expression cassette may constitute a good choice for the FA gene therapy trial in the near future (**Table 1**).

A significant stage in the process leading to the gene therapy of FA consists in demonstrating the feasibility and capacity to produce good manufacturing practice (GMP)-compliant, clinical-grade lentiviral vectors. The current GMP process to produce VSV-G-pseudotyped lentiviral vectors[39] is based on transient transfection of 293T cells in cell factory stacks, scaled up to 50 liters of viral stock per batch, followed by purification through several membrane-based and chromatographic steps, and leading to a 200-fold volume concentration and about 3-log^{10} reduction in protein and DNA contaminants. The purified particles are biologically active and nontoxic to cultured FA cells. Preliminary data show that a sufficient quantity to support pilot studies on small cohorts (~five patients) could be obtained per each run.

Thus, advancing FA gene therapy in Europe and worldwide is not hindered by roadblocks at the level of vector manufacturing. Funding issues need to be resolved, however, as it is estimated that the total costs of a single current GMP vector batch is about half a million euros ($ 710,000).

TARGET CELL POPULATION

Due to the extraordinary intolerance of FA HSC/HPCs to *in vitro* manipulation, protocols based on prolonged transductions of FA BM cells with γ-retroviral vectors cannot be used for FA cells.[40] The use of lentiviral vectors has improved the transduction efficiency of hematopoietic stem cell from FA patients after short incubation periods (<24 hours).[27,28] In addition, on a stem cell level, the short transduction of BM cells from FA mice markedly improved donor engraftment of genetically corrected cells,[41] which suggests a selective advantage of these cells *in vivo* that may similarly benefit human FA patients.

Conventional combination of growth factors (*i.e.*, Flt3-ligand, stem cell factor, and thrombopoietin, with or without granulocyte colony-stimulating factor) has been used in the *ex vivo* transduction of FA HSC/HPCs.[27–29] Improved cell viability has been achieved when cultures were maintained in hypoxia (*i.e.*, 5% instead of 21% ambient oxygen),[27,28] and when culture medium contained the antioxidant N-acetylcysteine[27] and the tumor necrosis factor α inhibitor etanercept.[28] Even though satisfactory transduction levels in whole FA BM have been obtained using GALV-TR pseudotyped lentivirus,[28] high vector titers have not been obtained so far under current GMP conditions. As purified hematopoietic progenitors (*i.e.*, CD34$^+$ cells) have been employed in all hematopoietic gene therapy trials, including the first FA trials with γ-retroviral vectors,[22,23] the panel proposed to use purified grafts from FA BM[22] or mobilized PB cells[42,43] as a cellular substrate for proposed stem cell gene therapy clinical trials in the near future (**Table 2**). Nevertheless, future clinical trials could evaluate whether accessory CD34$^-$ cells[44] or healthy MSCs[45] can significantly enhance the engraftment of corrected CD34$^+$ cells from FA patients. In addition, red blood cell depletion[28] might better preserve the repopulation ability of the autologous graft (**Table 2**).

Furthermore, we agreed that hematopoetic harvests should be performed in the early stages of the disease, when relatively high numbers of HSC/HPCs could be obtained. Hematopoetic cells harvested in the more advanced stages of the disease may be associated with few HSC/HPCs and an increased risk of accumulating mutations in HSC/HPCs. Importantly, HSC/HPCs mobilizers such as CXC chemokine receptor 4 antagonist AMD3100[46,47] have a potential to increase the numbers of HSC/HPC in peripheral blood in individuals for whom peripheral blood collection is preferred to the BM harvest (**Table 2**). For some FA patients HSC/HPCs may be harvested and stored for future use as determined by the onset

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Table 2 Cell populations to be considered in gene therapy of Fanconi anemia patients

Population	Advantages	Limitations
Purified CD34$^+$ HSCs (BM or mPB[a])	Extensively used in human hematopoietic transplantation and gene therapy trials.	The long-term repopulating ability of fresh FA CD34$^+$ cells has not been demonstrated so far (possibly due to altered expression of the CD34 antigen in FA HSCs).[28] In xenogenic transplantation models, the engraftment not only depends on the number of transplanted CD34$^+$ cells, but also of accessory CD34$^-$ cells.[44]
Purified CD34$^+$ cells + Support of allo MSCs	Healthy MSCs may improve the reconstitution of purified CD34$^+$ corrected cells.[45]	The long-term repopulating ability of fresh FA CD34$^+$ cells has not been demonstrated so far.[28] Costs for MSCs production.
RBCs-depleted BM (or mPB[a] cells)	Generalized genetic correction of hematopoietic and accessory cell populations.[28]	Not compatible with transduction protocols requiring high MOIs of current VSV-G LVs.
Enriched Lin$^-$ HSCs (BM or mPB[a])	Only differentiated hematopoietic cells are depleted from the graft.	Depletion systems for HSC enrichment have not yet been approved for human hematopoietic transplantation.

Abbreviations: allo, allogeneic; BM, bone marrow; FA, Fanconi anemia; HSC, hematopoietic stem cell; Lin, lineage; LV, lentiviral vector; MOI, multiplicity of infection; mPB, mobilized peripheral blood; MSC, mesenchymal stem cell; RBC, red blood cell; VSV-G, vesicular stomatitis virus.
[a]Potent mobilizers (*i.e.*, plerixafor) can be considered for HSC mobilization due to the limited efficacy of granulocyte colony-stimulating factor as a single mobilizing agent in FA patients.[42,43,46,67]

of hematological abnormalities, despite the fact that—when compared with fresh FA BM cells—the number of transduced HSC/HPCs obtained from cryopreserved FA BM cells is significantly reduced.[28] Nevertheless, transduction efficacies obtained with cryopreserved samples were adequate to correct the FA cellular phenotype, suggesting that gene-corrected cells could potentially rescue FA patients from their BM failure.

CLINICAL TRIAL

Over 70 patients with primary immunodeficiency have now been treated worldwide with stable reconstitution varying from long-term (>10 years) in X-linked severe combined immunodeficiency and adenosine deaminase deficient severe combined immunodeficiency, to conditions with shorter follow-up, as in chronic granulomatous disease.[21,48,49] For some indications, low intensity conditioning with alkylating agents such as busulfan or melphalan has provided sufficient myelosuppression to allow long-term engraftment of HSCs.[50,51] Clinical trials using lentiviral vectors and self-inactivating γ-retroviral vectors will provide important information with regard to safety and efficacy and have recently commenced for immunodeficiency, for therapy of human immunodeficiency virus infection, and for metabolic disease. Conditioning regimens are also being tailored to the specific disease, depending on the degree of HSC engraftment that is required for long-term effect, and on the level of growth and survival advantage conferred to corrected cell lineages. Despite the fact that the use of conditioning is controversial, the majority of the panel agreed that any discussion on the use of conditioning strategies for FA patients—either with alkylators such as cyclophosphamide or with alternative nongenotoxic approaches such as interferon-γ[8,52]—will only become meaningful if an initial phase 1 study shows that lentivirally corrected FA stem cells are safe to administer to patients without myelosuppression.

Notably, studies in large-animal models have shown that efficient gene transfer into long-term repopulating cells can be achieved with novel lentivirus and foamy virus vectors using short transduction cultures.[53,54] Based on these findings and the prevalence of FA complementation group A, we propose a gene therapy trial in patients with biallelic *FANCA* germ-line mutations with the following inclusion and exclusion criteria.

Major inclusion criteria:

1. FA demonstrated by a positive test for increased sensitivity to chromosomal breakage with mitomycin C or diepoxybutane, and FA complementation group A as determined by somatic cell hybrids, molecular characterization, western blot analysis, direct *FANCA* sequencing, or acquisition of mitomycin C resistance after *in vitro* transduction with a vector bearing the *FANCA* cDNA.
2. BM analysis demonstrating normal karyotype.

Major exclusion criteria:

1. Uncontrolled viral, bacterial, or fungal infection.
2. Patients with an HLA identical sibling donor.

SAFETY

Standard linear amplification-mediated (LAM)-PCR is currently the most sensitive method for comprehensive large-scale integration site (IS) analysis.[55] As LAM-PCR is dependent on restriction enzymes, multiple enzymes should be used to avoid having distinct integration sites masked.[56] Nonrestrictive LAM-PCR circumvents the restriction digest step, allowing retrieval of IS genome-wide.[56,57] Its lower sensitivity compared to LAM-PCR, however, may be an obstacle in pediatric gene therapy trials due to the limited DNA amount available in the lesser volumes of blood obtainable from small children.

In several γ-retroviral clinical gene therapy trials, a clustering of common IS (CIS) led to clonal dominance and even leukemia.[49,58–63] Though the occurrence of CIS might be used as prospective indicator for clonal imbalances, the clinical significance of isolated CIS is not always clear. The size of the clonal inventory and number of IS retrieved have to be taken into consideration and compared using mathematical and computational modeling to determine whether the degree of CIS occurrence over time is statistically significant.[64] In contrast to γ-retroviral vectors that integrate, preferably near transcriptional start sites, lentiviral

vectors show a potentially safer integration profile with regards to the probability of oncogene activation.[31,32,65] Even though the follow-up is much shorter than in the γ-retroviral clinical trials, to date no severe genotoxic side effects have been reported in lentiviral clinical trials.[26,63]

COLLABORATIONS

International harmonization of ethical and pharmaceutical regulatory requirements remains a significant challenge to establishing effective multi-centre gene therapy protocols and has been the subject of much recent discussion. This is important because it will allow the recruitment of suitable numbers of patients, more rapid application of technological advances, and comparison of different treatment protocols (for example, conditioning regimens). An increasing number of collaborative clinical trials are being conducted internationally with identical vectors and matched protocols, thereby providing a paradigm for true coordination and future multi-centre activity.

RECOMMENDATIONS

The meeting resulted in agreement that the outline of the first FA trial should involve rapid transduction of fresh or previously cryopreserved progenitor cells, followed by immediate intravenous infusion of the stem cell graft. This straightforward design is aimed at establishing the safety of FA cells modified with a lentivirally delivered *FANCA* gene and allows for accrual of FANCA$^{-/-}$ patients at several centers with seamless comparison of data.

NEXT STEPS

As new data are typically needed for new ideas, we expect that this approach will bring the field rapidly to the next generation of trials enriched by ideas that can only be gained from the clinical observation of treated FA patients.

Such modifications may include:

- FA-specific regulatory elements in the lentiviral vector,
- augmentation of the FA HSC/HPC transduction efficiency by novel lentiviral vector pseudotypes,
- inclusion of a suicide gene,
- HSC/HPC expansion and selection,
- HSC/HPC cryopreservation and serial infusions,
- conditioning before and/or after stem cell infusion,
- direct intra-BM infusion of gene-corrected cells,
- gene therapy for other FA complementation groups,
- risk stratification of FA patients, including on the basis of genotype, in an effort to tailor various treatment options (*e.g.*, androgens, allogeneic HCT, and gene therapy) and their timing to the specific needs of actual patients,
- gene therapy for extramedullar tissues (*e.g.*, oral mucosa), and
- lessons learned from the emerging field of cellular reprogramming.

These outcomes may be in the future, but the events that lead to them are not. Therefore, the ongoing task of this Working Group is to integrate all new relevant information from clinical and basic sciences to render this disease livable and ultimately a disease of the past.

OPEN QUESTIONS

Inevitably, many questions remain. In addition to FA-specific questions such as: "Will the revertant cell population fully replace the mutant hematopoiesis?" and "Will the loss of natural *FANCA* gene regulation be clinically relevant?," questions affecting the general field of gene transfer into hematopoietic progenitor cells are relevant to FA, such as: "Will host immune response target the gene-modified cells?," "How strongly will the genome integration data prospectively impact the clinical care of the patients?," "Is any disease-specific genome integration entirely random?," "Are sensitized cells or cancer-prone mice useful predictors of malignant transformation and tumorigenicity in humans?," and "Will late cancers due to cumulative acquisition of secondary mutations occur?". These uncertainties should not paralyze the field; rather they should energize us to design a coherent and pragmatic response to the formidable challenge of finding effective treatment for people with FA.

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