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Rapid Lentiviral Transduction Preserves the Engraftment Potential of *Fanca*$^{-/-}$ Hematopoietic Stem Cells

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Fanconi anemia (FA) is a rare recessive syndrome, characterized by congenital anomalies, bone marrow failure, and predisposition to cancer. Two earlier clinical trials utilizing γ-retroviral vectors for the transduction of autologous FA hematopoietic stem cells (HSCs) required extensive *in vitro* manipulation and failed to achieve detectable long-term engraftment of transduced HSCs. As a strategy for minimizing *ex vivo* manipulation, we investigated the use of a "rapid" lentiviral transduction protocol in a murine *Fanca*$^{-/-}$ model. Importantly, while this and most murine models of FA fail to completely mimic the human hematopoietic phenotype, we observed a high incidence of HSC transplant engraftment failure and low donor chimerism after conventional transduction (CT) of *Fanca*$^{-/-}$ donor cells. In contrast, rapid transduction (RT) of *Fanca*$^{-/-}$ HSCs preserved engraftment to the level achieved in wild-type cells, resulting in long-term multilineage engraftment of gene-modified cells. We also demonstrate the correction of the characteristic hypersensitivity of FA cells against the cross-linking agent mitomycin C (MMC), and provide evidence for the advantage of using pharmacoselection as a means of further increasing gene-modified cells after RT. Collectively, these data support the use of rapid lentiviral transduction for gene therapy in FA.

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INTRODUCTION

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 (reviewed in refs. 1,2).

The cumulative risk of bone marrow failure by age 40 in FA patients is 90%, with a median age of onset of 8 years.[3] FA patients are also prone to develop leukemia and cancer, particularly acute myelogenous leukemia and squamous cell carcinoma.[3,4] Currently, allogeneic stem-cell transplantation is the only curative treatment for bone marrow failure. Ten-year survival following matched sibling transplantation is >80%.[5] However, unaffected matched sibling donors are unavailable to the majority of the patients, and the results of alternative donor transplantation remain unsatisfactory.[6]

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.[7–10] 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.[11,12] However, these trials failed to achieve significant levels of engraftment of transduced cells and provided limited, if any, clinical benefit.

The success of these studies was hampered, in part, by the reduced content of HSCs and progenitors present in FA bone marrow.[11] In addition, murine FA HSCs have been shown to be prone to excessive apoptosis, and marrow containing these HSCs developed cytogenetic aberrations during *in vitro* culture under conditions that promote stem-cell/progenitor proliferation.[13] This is particularly relevant as a disease-specific challenge in FA gene therapy, because a common feature of conventional γ-retroviral transduction protocols employed in preclinical and all clinical gene transfer protocols to date involves cytokine prestimulation with the goal of inducing HSC proliferation, an essential prerequisite for γ-retroviral integration.[14] In earlier clinical gene therapy trials, *in vitro* culture of FA HSCs lasted up to 84 hours.[11,12]

Human immunodeficiency virus-1–derived lentiviral vectors appear capable of transducing nondividing cells, thereby permitting shortened *ex vivo* culture durations while maintaining gene transfer to long-term repopulating cells.[15] Effective transduction of long-term repopulating murine HSCs has been achieved with minimal cytokine exposure, and transduction as brief as 1–4 hours.[16,17]

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In this study, we applied a rapid transduction (RT) approach in a murine *Fanca*$^{-/-}$ transplant model. Several FA mouse models fail to fully mimic the human phenotype, particularly the progressive bone marrow failure seen in FA patients.[18] Engraftment defects have been described earlier in mice with a targeted disruption of *Fancc* or a hypomorphic mutation of the *FanD1* gene.[19,20] In addition, the duration of the culture has been shown to correlate inversely with the competitive engraftment fitness of *Fancc*$^{-/-}$ HSCs.[13] However, there are no earlier reports describing engraftment defects in the *Fanca*$^{-/-}$ mouse. The transplantation model described here demonstrates a profound engraftment defect in *Fanca*$^{-/-}$ HSCs under the stress of conventional prestimulation/conventional transduction (CT) conditions. Using this defect, we sought to develop two approaches to address the qualitative and quantitative limitation of HSCs during gene transfer protocols. We report significant preservation of engraftment function of FA HSCs, utilizing lentivirus vectors and a dramatically shortened *ex vivo* manipulation. In addition, we exploit coexpression of the drug-resistance gene O^6-methylguanine-DNA-methyltransferase P140K ($MGMT^{P140K}$)[21–25] to select for gene-corrected cells, thereby providing a mechanism to further expand low numbers of transduced and FA-pathway functional cells while selecting against FA-deficient cells.

RESULTS

Rapid transduction preserves engraftment of transduced *Fanca*$^{-/-}$ HSCs

In order to examine the biological consequences and potential benefits of RT in a *Fanca*$^{-/-}$ mouse model, a panel of lentiviral vectors (**Figure 1**) was utilized to transduce Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) bone marrow cells isolated from $CD45.2^+$ *Fanca*$^{-/-}$ or wild-type (wt) mice. After either CT or RT, the progeny cells derived from 2,000 LSK cells were injected into lethally irradiated $CD45.1^+$ wt mice. Observation endpoints were engraftment, transduction efficiency, and donor chimerism at 4 months after transplantation.

A striking difference in the rate of engraftment failure was observed between the two transduction methods (**Figure 2a**). Engraftment failure was defined by the presence of severe pancytopenia in moribund mice during days 10–21 after transplantation (**Supplementary Table S1**). Only 20 of 36 mice that received transplants of CT *Fanca*$^{-/-}$ LSK cells showed engraftment (55%, 95% confidence interval 0.381–0.721). By contrast, 36 of 36 mice that received transplants of RT *Fanca*$^{-/-}$ LSK showed engraftment and survived (100%, 95% CI 0.903–1.0). When bone marrow derived from wt mice was used, 11 of 12 (91%) CT and 12 of 12 RT mice survived (95% CI 0.715–1.0 and 0.735–1.0, respectively), a finding that supports the hypothesis that FA HSCs show increased sensitivity to *in vitro* manipulations. No deaths occurred after day 21 in any of the groups. Four months after the transplant, we investigated the donor chimerism in the peripheral blood of transplant recipients (**Figure 2b**). In mice that had received CT *Fanca*$^{-/-}$ cells, 26 ± 36% (mean ± SD) of peripheral blood leukocytes were donor-derived. In contrast, after RT, engraftment equivalent to those of wt cells was achieved with 93 ± 3% donor-derived cells (mean ± SD, $P < 0.01$ for comparison of *Fanca*$^{-/-}$ CT versus RT). Emphasizing the FA-specific cellular phenotype, only a marginally significant difference in donor chimerism was observed when bone marrow derived from wt mice was used (CT: 85 ± 12%, RT: 92 ± 2%, $P = 0.04$). In view of the fact that the results were not dependent on the vector construct (data not shown), the data include the results of all three vector constructs.

Next we analyzed the chimerism of gene-marked green fluorescent protein $(GFP)^+$ cells. While no significant difference in

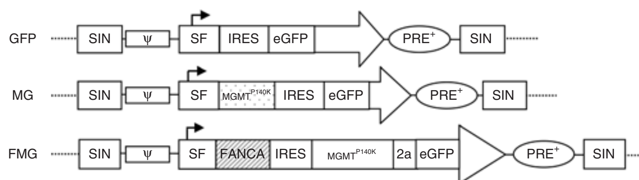

Figure 1 Schematic representation of the lentiviral vectors used in this study. Vectors are referred to as (i) GFP vector (mono-cistronic GFP-expressing construct), (ii) MG-vector (bi-cistronic $MGMT^{P140K}$/GFP construct), and (iii) FMG (tricistronic FANCA/$MGMT^{P140K}$/GFP construct). SIN, self-inactivating 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.

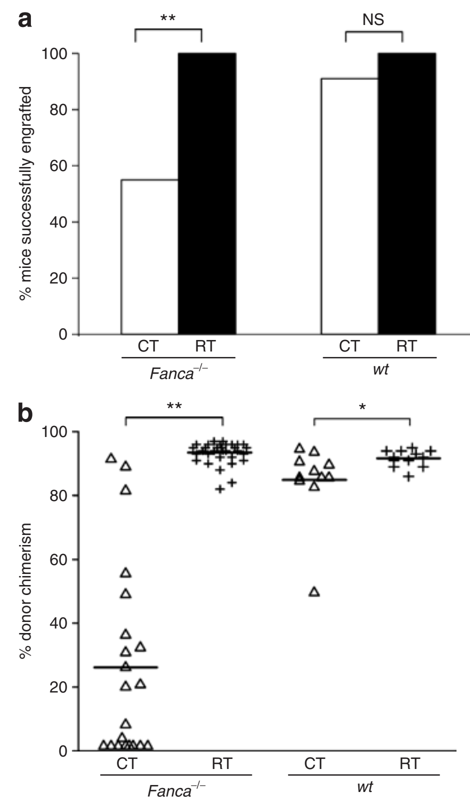

Figure 2 Engraftment rate and donor chimerism after conventional versus rapid transduction. The progeny of 2000 $CD45.2^+$ *Fanca*$^{-/-}$ or wild-type (wt) Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ cells were transplanted into lethally irradiated CD 45.1^+ wt mice. (**a**) Percentage of mice successfully engrafting after CT (white bars) versus RT (black bars); $N = 36$ and $N = 12$/condition in *Fanca*$^{-/-}$ and wt groups, respectively. $^{**}P < 0.01$ (Fisher's exact test); (**b**) Donor chimerism 4 months after the transplant. Each symbol represents a separate animal, bar represents the mean value for the group; *Fanca*$^{-/-}$, $N = 36$/condition, wt, $N = 12$/condition, $^{**}P < 0.01$, $^{*}P < 0.05$ (Wilcoxon rank-sum test). CT (open triangles), conventional transduction, RT (plus symbols), rapid transduction. NS, not significant.

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transduction rates was observed *in vitro* between RT and CT (data not shown), the superior engraftment of *Fanca*$^{-/-}$ HSCs with the use of RT led to significantly enhanced engraftment of gene-marked cells with the GFP vector (**Figure 3a**), as well as with the MG and FMG vectors (**Figure 3b** and **c**). Importantly, although equal numbers of mice in each cohort received transplantation (CT and RT), fewer mice were available for analysis in the CT cohorts because of engraftment failure. It is interesting that, no significant difference in the engraftment of gene-marked HSCs was observed in the recipients of CT vs. RT wt cells (GFP vector) (**Figure 3a**). The engraftment of gene-marked cells was stable for up to 8 months after the transplant (data not shown). We conclude that RT of *Fanca*$^{-/-}$ HSCs significantly preserves the engraftment potential of these cells and, in contrast to CT, leads to stable engraftment of gene-marked HSCs.

Rapid transduction results in genetic modification of long-term repopulating *Fanca*$^{-/-}$ HSCs

In order to investigate further whether gene marking occurs at the level of a long-term repopulating HSC, we performed secondary transplantation of stably engrafted GFP$^+$ *Fanca*$^{-/-}$ bone marrow mononuclear cells (BMMCs) from primary recipients 3 months after the transplant. In all cases, *Fanca*$^{-/-}$ LSK cells had been RT with the GFP vector. Approximately 5×10^5 GFP$^+$ BMMCs (CD45.2$^+$) were isolated from the primary recipients by FACS sorting, mixed with 1×10^5 fresh CD45.1$^+$ wt competitor BMMCs, and transplanted into lethally irradiated CD45.1$^+$ wt recipients. Twelve weeks after the transplant, engraftment of GFP$^+$ cells was noted in all eight recipient animals (**Figure 4a**), with the levels of GFP$^+$ cells varying from ~10 to >80%. We next investigated the contribution of gene-marked (GFP$^+$) peripheral blood leukocytes to the T-cell (CD3$^+$), B-cell (B220$^+$), and granulocyte (MAC-1$^+$) subpopulations in both primary and secondary recipients (**Figure 4b**). Gene-marked leukocytes contributed to all lineages without a statistical difference between primary and secondary recipients (primary recipients $N = 9$, secondary recipients $N = 6$). We conclude that RT occurred at the level of the long-term repopulating HSC.

Tricistronic vector construct enables pharmacoselection and confers correction of cellular *Fanca*$^{-/-}$ phenotype

In order to provide a method of expanding a limited number of transduced HSCs, we as well as others have exploited the use of drug selection markers and pharmacoselection of HSCs. The utility of

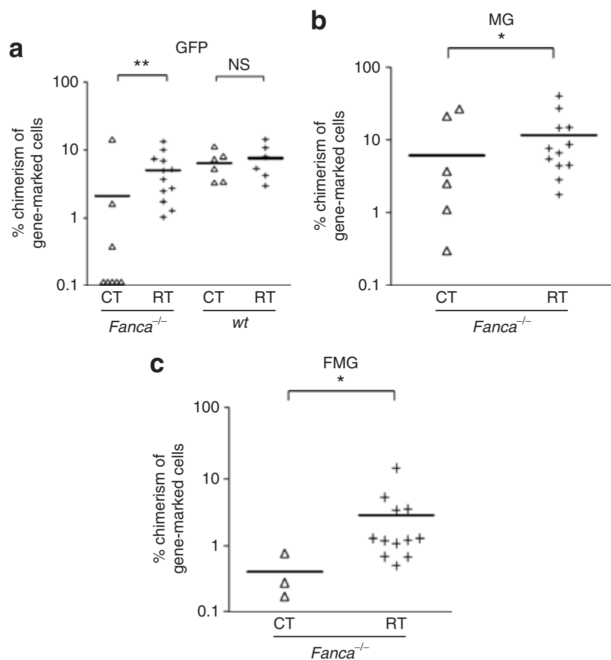

Figure 3 Chimerism of long-term engrafted gene-marked (GFP$^+$) cells. *Fanca*$^{-/-}$ or wt Lin$^-$ Sca-1$^+$ c-Kit$^+$ cells were employed for transduction using CT (open triangles) or RT (plus symbols). Equal numbers of mice received the transplants in each cohort. However, because of engraftment failure, fewer CT than RT animals were available for analysis. Flow cytometry was performed on peripheral blood after red cell lysis; (**a**) GFP vector analyzed at 12–16 weeks after transduction; (**b**) MG-vector analyzed at 16 weeks after transduction; (**c**) FMG vector analyzed at 16 weeks after transplantation. $^{**}P < 0.01$, $^{*}P < 0.05$, NS, not significant (Wilcoxon rank-sum test). CT, conventional transduction; FMG vector, tricistronic FANCA/MGMT^{P140K}/GFP construct; GFP, green fluorescent protein; GFP vector, mono-cistronic GFP-expressing construct; MGMT^{P140K}, P140K mutant of O^6-methylguanine-DNA-methyltransferase; MG-vector, bi-cistronic MGMT^{P140K}/GFP construct; RT, rapid transduction.

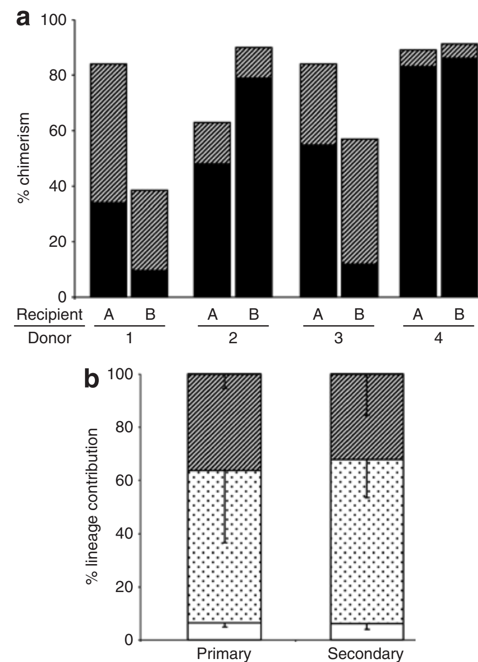

Figure 4 Secondary transplantation and analysis of lineage contribution of gene-marked cells following rapid transduction of Fanca$^{-/-}$ Lin– Sca-1+ c-Kit+ cells with the green fluorescent protein (GFP)-vector. (**a**) CD45.2$^+$ GFP-positive bone marrow mononuclear cells were isolated from engrafted primary transplant recipients and transplanted into four pairs (denoted A and B) of lethally irradiated CD45.1$^+$ wild-type secondary recipients. Peripheral blood was analyzed for donor chimerism and GFP$^+$ cells 12 weeks after the transplant, using flow cytometry. Black bars, GFP-positive donor-derived cells; hatched bars, GFP-negative donor-derived cells; (**b**) contribution of gene-marked (GFP$^+$) peripheral blood leukocytes to T cells (CD3$^+$, white bars), B cells (B-220$^+$, dotted bars) and granulocytes (MAC-1$^+$, hatched bars) in engrafted primary and secondary recipients (12–16 weeks after the transplant). $N = 9$ (primary recipients) and $N = 6$ (secondary recipients). The data are presented as mean values ± SD.

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overexpression of the P140K mutant of MGMT^{P140K} in the presence of O^6-benzylguanine (an inhibitor of endogenous MGMT) and chemotherapeutic agents such as temozolomide, has been demonstrated earlier, by us as well as by others.[21–25] In order to assess the function of the *FANCA* (FA-A) gene in a tricistronic vector that coexpresses FANCA, MGMT^{P140K}, and enhanced GFP (FMG, **Figure 1**), this vector was used for transducing a human FANCA-defective Epstein-Barr virus–transformed lymphoblast cell line (LCL). Cell cycle analysis was performed after exposure to the DNA-damaging drug melphalan. The percentage of LCLs exhibiting a G2/M arrest was reduced by 24% after transduction with the FMG vector, when compared with the control GFP vector (**Figure 5a**). This demonstrates FA cellular phenotypic correction.[26]

In order to assess whether correction of the cellular FA phenotype also occurs in long-term engrafted cells after RT, GFP$^+$ BMMCs were isolated from the transplant recipients at 4 months after the transplant, and progenitor colony survival was analyzed in the presence or absence of either 5 or 10 µmol/l mitomycin C (MMC) (**Figure 5b**). As expected, untransduced *Fanca*$^{-/-}$ cells (FA in **Figure 5b**) showed hypersensitivity to MMC, and this treatment significantly reduced the survival of progenitor cells at these concentrations of the drug. The number of progenitor colonies, formed by the FMG-transduced *Fanca*$^{-/-}$ cells and surviving in the presence of MMC, was significantly higher when compared with nontransduced bone marrow cells and, with correction using an FMG vector, the survival rate achieved was comparable to that in wt animals.

Next we investigated the potential for MGMT^{P140K}-mediated pharmacoselection in BMMCs obtained from primary transplant recipients. Bone marrow was harvested 4 months after the transplant, from mice reconstituted with *Fanca*$^{-/-}$ cells that had been RT with the MG and FMG vectors (**Figure 1**). BMMCs were then exposed to O^6-benzylguanine and temozolomide. A single drug exposure resulted in potent selection of donor (CD45.2-derived) GFP$^+$ cells (14- and 11.5-fold, for MG and FMG, respectively) after 6 days (**Figure 5c**). As expected, no selection occurred in GFP-transduced control bone marrow cells (data not shown).

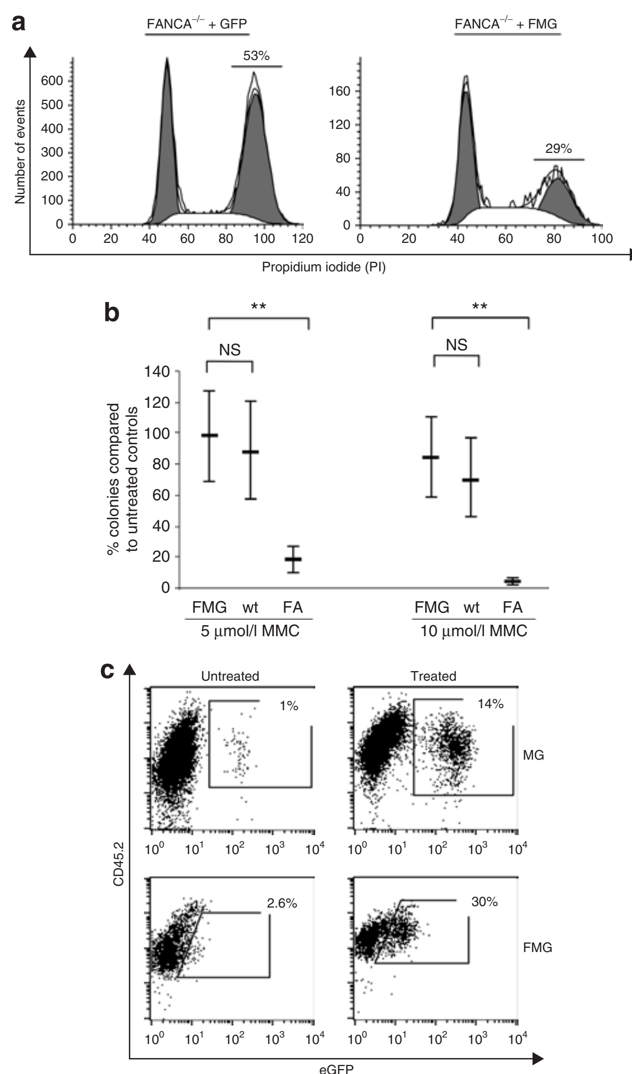

Figure 5 Correction of Fanconi anemia (FA) cellular phenotype and pharmacoselection. (**a**) A human FANCA-defective Epstein-Barr virus–transformed lymphoblast cell line was transduced and exposed to melphalan. Cell cycle analysis reveals the percentage of cells in the G2/M arrest after transduction with a green fluorescent protein (GFP)-vector (control) and FMG; (**b**) bone marrow mononuclear cells (BMMCs) harvested from engrafted transplant recipients 4 months after the transplant were sorted for GFP$^+$ cells by fluorescence-activated cell sorting, and plated in methylcellulose in the presence of 0, 5, or 10 µmol/l mitomycin C (MMC). The data represent mean values ± 95% confidence interval, $N = 3$. **$P < 0.01$ (FMG versus *Fanca*$^{-/-}$), NS, not significant (*F*-test, linear mixed model), FA, *Fanca*$^{-/-}$; (**c**) BMMCs harvested from primary transplant recipients as in (**b**) were exposed in bulk culture to 20 µmol/l O6-BG and 12.5 µg/ml temozolomide. Flow cytometry for CD45.2 allophycocyanin and GFP was performed 6 days after drug treatment ($N = 3$). Number indicates the percentage of GFP-expressing cells. FMG, tricistronic FANCA/MGMT^{P140K}/GFP construct; MGMT^{P140K}, P140K mutant of *O*^{6}-methylguanine-DNA-methyltransferase; MG, bi-cistronic MGMT^{P140K}/GFP construct; PI, propidium iodide.

Analysis of proviral copy number in colony forming units granulocyte–macrophage colonies

Because of the high multiplicity of infection utilized in the RT protocol[16,17] and the potential risks of insertional mutagenesis, we next assessed the number of proviral integrations in transduced cells. GFP$^+$ BMMCs from mice that had received transplants of all three vectors (**Figure 1**) were isolated using flow sorting 3 months after the transplant, and plated in methylcellulose under conditions yielding granulocyte-macrophage colonies. After 7 days, individual colonies were "plucked" and the number of proviral copies was determined using quantitative real-time PCR (**Figure 6**). In the case the GFP vector, the proviral copy number was 4 ± 1.6 (102 colonies derived from two mice, mean value ± SD) (**Figure 6a**). For the MG and FMG vectors, the copy numbers were 3 ± 1.4 and 3 ± 2, respectively (**Figure 6b** and **c**; for each vector, 45 colonies were derived from three mice and analyzed).

DISCUSSION

The studies reported here were motivated by the lack of evidence of long-term clinical benefit in the data from earlier clinical gene therapy trials for FA.[11,12] FA is an attractive candidate disease for the therapeutic use of gene transfer methodology. Bone marrow failure occurs almost universally in FA patients and is the major cause of morbidity and mortality during childhood and adolescence.[3] Currently, allogeneic stem-cell transplantation is the only curative therapy option for bone marrow failure. While outcomes

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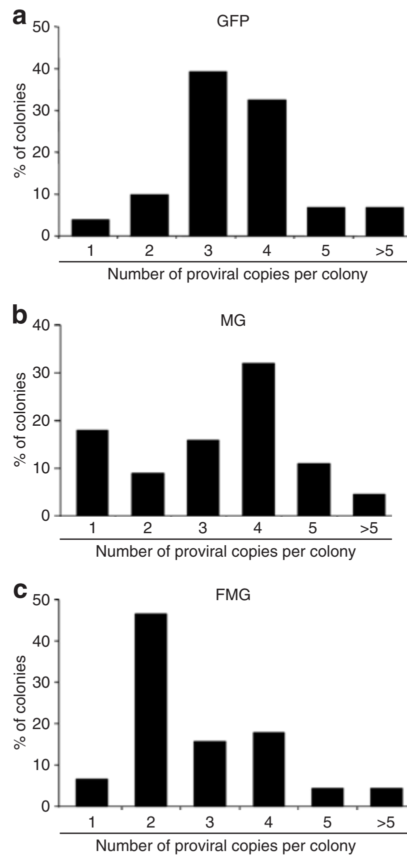

Figure 6 Analysis of proviral green fluorescent protein (GFP) copy number. Individual GFP$^+$ colony forming units granulocyte-macrophage colonies derived from engrafted transplant recipients >3 months after the transplant were plucked from methylcellulose and interrogated using quantitative real-time PCR for proviral eGFP copy number. (**a**) GFP vector (102 colonies derived from two mice), (**b**) MG vector (45 colonies from 3 mice), (**c**) FMG vector (45 colonies from 3 mice). The graph shows the percentage of colonies with 1 to >5 copies/cell. FMG vector, tricistronic FANCA/MGMT^{P140K}/GFP construct; GFP vector, mono-cistronic GFP-expressing construct; MGMT^{P140K}, P140K mutant of *O*^{6}-methylguanine-DNA-methyltransferase; MG-vector, bi-cistronic MGMT^{P140K}/GFP construct.

of this procedure continue to improve, morbidity and mortality remain high, particularly in the absence of an unaffected matched sibling donor.[6] This is partly because of the complications of graft-versus-host disease and graft failure, and also because of acute regimen-related toxicities. Genetic correction and reinfusion of autologous HSCs may potentially offer hope to a selected group of patients in the future. It has been hypothesized that the genetic correction of a small subset of FA HSCs will confer a selective advantage to these cells, allowing for *in vivo* expansion of corrected HSCs. This hypothesis is based largely on the observation that a few FA patients have been reported to show evidence of acquired genetic reversion in myeloid lineages associated with improved hematopoietic function.[27,28] Moreover, a competitive advantage of gene-corrected HSCs/Ps has been described in murine models of Fancc deficiency.[29,30] However, it remains unclear whether this effect is relevant to human gene therapy, and whether it is possible to rely upon its ability to expand a limited number of engrafted and genetically corrected cells in the manner demonstrated in successful immunodeficiency gene therapy trials.[31–33]

In earlier clinical gene therapy trials relating to FA,[11,12] proviral sequences were detected in a maximum of 0.1% of the peripheral blood mononuclear cells for <4 months after infusion of HSCs. This was despite achieving, *in vitro*, a satisfactory 30–40% transduction rate of defined hematopoietic cell populations using conventional γ-retroviral transduction procedures. The lack of engraftment of gene-modified autologous HSCs may be attributed to by a combination of several factors, including relatively low cell doses on account of disease-specific progressive loss of HSCs, lack of conditioning of the recipient, and prolonged (up to 84 hours) *ex vivo* manipulation of CD34$^+$ cells during the γ-retroviral transduction procedure. Relating as it does to the number and function of transduced FA HSCs, *in vitro* culture of HSCs under conditions that induce cell proliferation (as are required for γ-retroviral transduction) has been described, in at least one report of the *Fancc*$^{-/-}$ murine model, as leading to a loss of competitive repopulation ability, to apoptosis, and to cytogenetic aberrations.[13] In addition, these data may call into question the putative "selective advantage" of gene-corrected FA HSCs.

While most murine knockout models of FA fail to mimic important aspects of the human phenotype, including the progressive pancytopenia and bone marrow hypoplasia,[18] engraftment defects have earlier been demonstrated in mice with a targeted disruption of *Fancc* or a hypomorphic mutation of the *Fand1* gene.[19,20] Under the conditions used here, the engraftment defect of *Fanca*$^{-/-}$ HSCs was striking, leading to graft failure and resulting in death in approximately one-third of the transplant recipients and diminished donor chimerism in the surviving mice. Depletion of repopulating HSCs during *in vitro* culture has been described earlier in *Fancc*$^{-/-}$ HSCs[13] and is likely to have contributed to the effect observed in this study. Notably, this 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.

While successes in X-linked severe combined immunodeficiency and adenosine deaminase–severe combined immunodeficiency gene therapy trials[31–33] were likely to have been, at least in part, the result of the strong *in vivo* selection of gene-corrected cells, it is not yet clear whether such selective pressure exists for FA.[11] MGMT has emerged as a powerful selectable marker in HSCs and has been used successfully in some large animal models,[34] as reviewed by Milsom *et al.*[25] In the light of the modest gene transfer rates achieved with the RT protocol, we generated bi- and tricistronic vectors that express the O^6-benzylguanine resistant MGMT^{P140K} mutant in conjunction with the GFP and FANCA gene. Both vectors provided powerful selection of long-term engrafted bone marrow with a single exposure to the drug *in vitro*. It is important to note that selection is achieved despite low initial gene marking, a scenario that is likely to occur in clinical gene therapy trials. However, caution should continue to be exercised

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in selecting this approach for clinical application in FA patients, because it involves the use of a DNA-damaging agent in the setting of a disease-specific, increased genomic instability phenotype. Nongenotoxic selection approaches, such as the use of inhibitory cytokines,[35,36] may ultimately prove more feasible in clinical trials.

RT was performed with a significant excess of viral particles (multiplicity of infection of 100) as described in earlier reports.[16,17] However, transduction rates were modest overall, ranging between 1 and 10%. A detailed analysis was carried out of the number of proviral copies in 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. Earlier reports of rapid lentiviral transduction of wt HSCs had indicated an average proviral copy number of 1.5 and 3, respectively.[16,17] However, in these previous analyses, whole bone marrow (including nontransduced cells) rather than single colonies were investigated, possibly leading to an underestimation of the true copy numbers. While there is some emerging evidence that lentiviral transduction may pose less risk for insertional mutagenesis,[37] additional adjustment of the transduction conditions with the goal of transducing more stem cells with fewer copies per cell are clearly indicated.

Collectively, these data underline the promise held by rapid lentiviral transduction of FA HSCs. Further study of this approach in the context of human HSCs is warranted in order to facilitate translation into a clinical trial.

MATERIALS AND METHODS

Vector constructs. The lentiviral vector used[38,39] 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.[40] A panel of expression vectors was generated (**Figure 1**), including (i) a vector expressing the enhanced GFP gene (referred to as GFP vector), (ii) the MGMT $MGMT^{P140K}$ (ref. 41) and GFP genes (MG vector), and (iii) a tricistronic vector containing the *Fanca*, $MGMT^{P140K}$, and GFP genes (FMG vector).

Production of lentiviral vectors. Lentiviral vectors, pseudotyped with the vesicular stomatitis virus glycoprotein, were generated by transient transfection of 293T cells, and subsequently concentrated using ultracentrifugation as described earlier.[38,42] Titers ranged between 3×10^7 and 3.7×10^8 transducing units/ml.

Mice used in the experiments. All the mice were maintained in a specific pathogen-free environment and the experiments were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Research Foundation. C57BL/6J mice (CD45.2+ wt) and B6.SJL-$Ptprc^a$ $Pepc^b$/BoyJ mice (CD45.1+ wt) were obtained from Jackson Laboratories (Bar Harbor, ME). $Fanca^{-/-}$ mice have been described elsewhere.[18]

Isolation, transduction, and transplantation of HSCs. LSK bone marrow cells were isolated as described earlier.[43] The cells were transduced on CH296 fibronectin (4 μg/cm^2; Takara Bio, Otsu, Japan[44]) for 4 hours (rapid) or 94 hours (conventional) at a multiplicity of infection of 100 for RT and 20 for CT. RT[16,17] was performed in the presence of 50 ng/ml of recombinant rat stem-cell factor and 100 ng/ml of megakaryocyte growth and development factor (provided by Amgen, Thousand Oaks, CA), whereas CT was performed in the presence of 100 ng/ml recombinant rat stem-cell factor, human interleukin-11 (provided by Genetics Institute, Cambridge, MA), human Flt-3L, and 20 ng/ml murine interleukin-3 (Peprotec, Rocky Hill, NJ). After transduction, the cells were washed with phosphate-buffered saline and dissociated from fibronectin using cell stripper solution (Mediatech, Herdon, VA). The progeny of 2,000 LSK cells were injected through the tail veins of lethally irradiated (1,175 cGy, 56 cGy/min, Cs^{135}, split dose) CD45.1+ wt mice. After the transplantation, the moribund mice were analyzed by gross necropsy, complete blood count, bone marrow cellularity, and colony forming unit spleen. The mice were deemed to have succumbed to engraftment failure if death occurred in the 10–21 days period after transplantation, or if severe cytopenia was documented on the complete blood count.

Flow cytometry. Flow cytometry was performed using a FACSCalibur and FACSCanto flow cytometer (Becton Dickinson, San Jose, CA). Engraftment chimerism and gene marking in transplant recipients was determined by performing red cell lysis (BD Pharm Lyse; BD Bioscience Pharmingen, San Jose, CA) and staining peripheral blood leukocytes with anti-mouse allophycocyanin-labeled CD45.2 antibody (Pharmingen, San Diego, CA). The percentages of viable 7AAD (Invitrogen, Carlsbad, CA) donor-derived CD45.2+ and CD45.2+/GFP+ leukocytes were determined. In order to assess the contribution of transduced (GFP+) cells to major leukocyte lineages, costaining with phycoerythrin-labeled mouse monoclonal antibodies against the CD3 epsilon (T lymphocytes), B220 (B lymphocytes), and MAC-1 (granulocytes) epitopes (all antibodies purchased from Pharmingen) was performed.

Clonogenic assays and PCR analysis of colony forming units granulocyte-macrophage colonies. Bone marrow was harvested from mice 4 months after the transplantation, and low density BMMCs were isolated by density gradient centrifugation using Histopaque 1.083 g/cm^3 (Sigma, St Louis, MO). For clonogenic assays, GFP-positive cells were sorted (FACSVantage; Becton Dickinson, San Jose, CA), and 2,000 or 10,000 mononuclear cells were plated out in triplicate in methylcellulose (Methocult; Stem Cell Technologies, Vancouver, Canada) supplemented with 30% fetal calf serum, 2 mmol/l L-glutamine (Hyclone, Logan, UT), 200 U penicillin/streptomycin (Hyclone), 100 μmol/l β-mercaptoethanol (Thermo Fisher, Waltham, MA), 1% bovine serum albumin (Roche, Indianapolis, IN), and 100 ng/ml recombinant rat stem-cell factor, 100 ng/ml mIL3, 4 U/ml Epogen (Amgen). Five or ten μM of MMC (Bedford Laboratories Bedford, OH) were added where appropriate. The plates were incubated for 7 days in a humidified atmosphere (37 °C, 5% CO_2), and colonies were enumerated on day 7. At that time-point, nontreated individual progenitor-derived colonies were also plucked as described elsewhere.[45] Quantitative real-time PCR was performed on DNA isolated from individual colonies, as described earlier.[46] Probe sets were directed against both enhanced GFP (5′-ACTACAACA GCCACAACGTCTATATCA-3′, 5′-GGCGGATCTTGA AGTTCACC-3′, and 5′-FAM-CCGACAAGCAGAAGAACGGCATCA-3′-TAMRA as a probe) and the murine *ApoB* genomic sequence (5′-CGTGGGCTCCAG CATTCTA-3′, 5′-TCACCAGTCATTTCTGCCTTTG-3′, and 5′-VIC-CCT TGAGCAGTGCCCGACCATTC-3′-TAMRA).

In vitro *pharmacoselection.* For *in vitro* selection, BMMCs were cultured at a density of 1×10^6 cells/per well in a six-well plate in Iscove's modified Dulbecco's medium (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (Omega Scientific, Tarzana, CA), in the presence of 100 ng/ml recombinant rat stem-cell factor (Amgen), human interleukin-11 (provided by Genetics Institute, Cambridge, MA), human Flt-3L, and 20 ng/ml murine interleukin-3 (Peprotec, Rocky Hill, NJ). For pharmacoselection, the cells were treated with 20 μmol/l O^6-Benzylguanine (Sigma) for 1 hour, followed by exposure to temozolomide (Chemodex, St Gallen, Switzerland) at a concentration of 12.5 μg/ml. The cells were analyzed using flow cytometry 6 days after drug treatment.

Transduction of $FANCA^{-/-}$ LCL and cell-cycle analysis. Transduction and cell-cycle analysis of Epstein-Barr virus–transformed cell lines from

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FANCA$^{-/-}$ patients has been described elsewhere.[26] Briefly, transduction of LCLs was performed on nontissue-culture treated six-well plates coated with the fibronectin fragment CH296 (1 μg/cm^2; Takara Shuzo, Otsu, Japan). In order to induce cell cycle arrest, ~1×10^6 cells were plated in culture medium containing 0.3 μg/ml melphalan (Sigma). Analysis of the cell cycle was performed using 50 μg/ml propidium iodide (Sigma). Flow cytometry was performed using a FACSCanto flow cytometer (BD, Franklin Lakes, NJ). Linear propidium iodide emission of propidium iodide was collected for 10,000 GFP$^+$ cells. An analysis of the percentage of cells in each phase of the cell cycle was performed using MODFIT-LT software (Verity Software House, Topsham, ME).

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SUPPLEMENTARY MATERIAL

Table S1. Hematologic analysis indicating engraftment failure of transplanted mice.

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