

Stem Cell Collection and Gene Transfer in Fanconi Anemia

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Fanconi anemia (FA) is a rare genetic syndrome characterized by progressive bone marrow failure (BMF), congenital anomalies, and a predisposition to malignancy. Successful gene transfer into hematopoietic stem cells (HSCs) could reverse BMF in this disease. We developed clinical trials to determine whether a sufficient number of CD34⁺ stem cells could be collected for gene modification and to evaluate the safety and efficacy of HSC-corrective gene transfer in FA genotype A (*FANCA*) patients. Here, we report that FA patients have significant depletion of their BM CD34⁺ cell compartment even before severe pancytopenia is present. However, oncoretroviral-mediated *ex vivo* gene transfer was efficient in clinical scale in FA-A cells, leading to reversal of the cellular phenotype in a significant percentage of CD34⁺ cells. Re-infusion of gene-corrected products in two patients was safe and well tolerated and accompanied by transient improvements in hemoglobin and platelet counts. Gene correction was transient, likely owing to the low dose of gene-corrected cells infused. Our early experience shows that stem cell collection is well tolerated in FA patients and suggests that collection be considered as early as possible in patients who are potential candidates for future gene transfer trials.

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INTRODUCTION

Fanconi anemia (FA) is a rare inherited syndrome characterized by progressive bone marrow failure (BMF), congenital anomalies, and a predisposition to malignancy (reviewed by Taniguchi and D'Andrea¹). FA exhibits extensive genetic heterogeneity with at least 12 complementation groups, and 11 genes are identified. FA genotype A (FA-A) is the most common complementation

group, with biallelic mutations in *FANCA* accounting for approximately 60% of all FA patients.² The cumulative risk of BMF in FA patients is 90% by 40 years of age, with a median age of onset of BMF of 8 years.² Hematopoietic stem cell (HSC) transplantation is curative of BMF with a 5-year survival rate after a matched sibling transplant of approximately 85%.³ However, less than 25% of patients have an unaffected, matched sibling donor. Matched unrelated donor transplants increase donor availability, although regimen-related FA-specific conditioning toxicity and excessive graft rejection have a limited overall survival of 30–60% in reported series.^{4–6}

Viral vector-mediated gene transfer has shown promise in other monogenic diseases affecting the immune and blood systems.^{7–9} Expression of complementing FA cDNA in cells from FA patients corrects excessive chromosomal breakage, G2 cell cycle arrest, and increased mitomycin-C (MMC) sensitivity that are cellular phenotypes of the disease allowing improved growth of BM progenitor cells *in vitro*.^{10–13} Such gene transfer has successfully corrected the cellular defects in *Fanc*^{-/-} mice both *in vitro* and *in vivo*.¹⁴ As the genetic defects that cause the Fanconi phenotype appear to affect HSCs or long-lived progenitor cells with multilineage potential and significant proliferative capacity, it is possible that genetic correction of even a limited number of these cells, if accompanied by a selective growth advantage *in vivo* over uncorrected cells, could result in oligoclonal repopulation of the BM compartment and reversal or delay of aplasia. Indeed, rare FA patients have been diagnosed with a germline cellular defect (disease-associated mutation in an FA gene with abnormal chromosomal fragility testing in fibroblasts) but with normal hematopoiesis and normal blood/marrow MMC sensitivity. This clinical phenotype is hypothesized to be due to spontaneous “gene correction” in the hematopoietic cell compartment, suggesting that therapeutic-induced correction and expansion of stem cell clones could be feasible.^{15–18}

Unfortunately, by the time therapeutic intervention for pancytopenia is indicated, the number of target HSCs is almost

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certainly critically depleted. In addition, FA cells appear to be prone to excessive apoptosis during *in vitro* manipulations that are currently required for efficient gene transfer.¹⁹ We hypothesized that patients evaluated before the onset of severe pancytopenia may have sufficient stem cell numbers to allow collection and cryopreservation of HSCs for future autologous use. This approach could also enhance the success of gene therapy studies by preserving a large number of CD34⁺ targets for gene correction. We thus developed tandem clinical trials to determine whether a sufficient number of CD34⁺ stem cells could be collected and purified for future clinical use and, in parallel, a clinical trial to evaluate the safety and efficacy of HSC-corrective gene transfer in complementation group A (FA-A) patients (Figure 1).

Here, we report that FA patients have significant depletion of their BM CD34⁺ cell compartment even early in their disease progression. Enhanced methods of oncoretroviral-mediated *ex vivo* gene transfer were very efficient in clinical scale correction of FA-A cells. Gene transduction was successful at reversing the cellular phenotype of a significant percentage of CD34⁺ cells. Re-infusion of these gene-corrected products in a limited number of patients was shown to be safe and well tolerated with transient improvements in the recipients' hemoglobin (Hb) and platelet counts, however likely unrelated to the transfer of the FA-A cDNA. Thus, our early experience shows that stem cell collection is well tolerated in FA patients and suggests that such collection and cryopreservation should be considered as early as possible in the disease process in those patients who are potential candidates for gene therapy trials or other stem cell modifications in the future.

RESULTS

Cytopenia and BM cellularity in FA patients

From February 2002 until February 2006, 54 new FA patients were evaluated in the Fanconi Anemia Comprehensive Care Center at Cincinnati Children's Hospital Medical Center. Thirty-eight patients underwent a BM aspirate and biopsy as part of their clinical evaluation and provided a sample of BM for research studies. Of these research samples, 21 patients aged 1.5–26 years had peripheral blood (PB) cell counts showing mild cytopenias (defined as an absolute neutrophil count > 750/mm³, an Hb > 8.0 g/dl, or platelets > 30,000/mm³) and no evidence of myelodysplastic syndrome or leukemia. Eighteen of these patients

had BM samples analyzed for cellularity and CD34⁺ cell content (Table 1). FA patients had a 2-fold reduction in marrow cellularity and a 6-fold reduction in CD34⁺ cell content compared with normal adults. BM progenitor content was also evaluated on aspirate samples in 14 patients using *in vitro* assays and were also significantly reduced in these patients (Table 1).

CD34 collection in FA patients

Seven FA patients enrolled in the collection study and underwent the CD34⁺ cell collection process (Table 2). BM aspirate and the estimated weight 5 years in the future predicted that a BM harvest would not collect close to the target number of 2×10^6 CD34⁺ cells/kg (future weight) in three patients. Granulocyte colony-stimulating factor (G-CSF) mobilization and collection was recommended to these families. One family (patient 101) elected to undergo BM collection without G-CSF mobilization. Two patients (patients 102 and 105) received 16 µg/kg of G-CSF for 4 days followed by an additional 4 days of G-CSF at 32 µg/kg. G-CSF was well tolerated. PB absolute neutrophil counts increased from 780 cells/mm³ to 14,000 cells/mm³ in patient 102 and from 1,000/mm³ to 11,700/mm³ in patient 105. The protocol mobilization target of five CD34⁺ cells/µl of PB was not attained in either patient even after a total of 8 days of high-dose G-CSF; therefore, apheresis was not performed. These patients subsequently had marrow collections performed 7–14 days after G-CSF was stopped.

BM collection in the remaining patients was well tolerated. One patient developed a localized infection at one marrow aspirate site requiring oral antibiotics. Hemoglobin dropped by an average of 4.5 g/dl (range 3.4–5.1 g/dl) in the three patients who did not receive a blood transfusion (Table 3). Hemoglobin returned to baseline within 1 month of their BM harvest. Four patients received a 10 ml/kg packed erythrocyte transfusion peri-operatively based on low pre-harvest Hb.

The total number of nucleated cells in the BM collections, which ranged from 1.4 to 3×10^8 cells/kg (Table 3), was within the range predicted based on the marrow aspirate analysis at study entry. However, the frequency of CD34⁺ cells in the marrow collections, which ranged from 0.14 to 1.5%, was lower than the entry marrow aspirate analysis in six of seven patients. Although the target CD34⁺ cell dose of 2×10^6 /kg future weight was not obtained in any patient, three of four patients achieved >80% of the collection goal based on future weight and all four attained a collection of 2×10^6 CD34 cells/kg based on their current weight (Table 3 and Figure 2). Both patients treated with G-CSF and then subsequently collected by BM harvest, in contrast to those who did not receive G-CSF, yielded CD34⁺ cell collections that exceeded the predicted collection based on the entry marrow aspirate analysis ($P < 0.05$, G-CSF treated versus not treated) (Figure 2).

CD34 cell isolation in FA patient collection

CD34⁺ cell isolation was performed on collections with $> 1 \times 10^6$ CD34⁺ cells/kg future weight or in subjects participating in the gene transfer study for FA-A. Collections

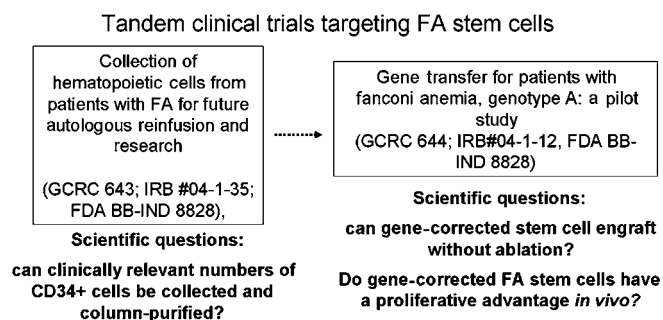


Figure 1 Schema for tandem trials targeting FA stem cells.

Table 1 BM cellularity and CD34⁺ cell content in FA patients without severe PB cytopenias

	MNC/ml ($\times 10^6$)	CD34 ⁺ content (%)	Estimated CD34 ⁺ cells/kg ($\times 10^6$) ^a	CFUs/10 ⁵ MNC
FA patients (N=18)	4.1 \pm 4.1*	1.0 \pm 1.1*	1.0 \pm 1.4*	39 \pm 27*
	Median: 2.7	Median: 0.6	Median: 0.4	Median: 37
	Range: 0.4–16.5	Range: 0.1–4	Range: 0.01–5.2	Range: 9–95 (N=14)
Normal donors (N=56)	8.1 \pm 7.2	3.0 \pm 2.6	5.9 \pm 8.9	206 \pm 43
	Median: 7.1	Median: 2.4	Median: 2.4	Median: 200
	Range: 0.2–33	Range: 0.2–14.6	Range: 0.03–44	Range: 164–260 (N=5) *P<0.001
	*P<0.03	*P<0.006	*P<0.03	

BM, bone marrow; CFU, colony-forming unit; FA, Fanconi anemia; MNC, mononuclear cell; PB, peripheral blood. ^aBased on a theoretical 20 ml/kg bone marrow harvest.

Table 2 BM cellularity of FA collection (FA Col) patients at study entry

No. of patients	Age (years)	Weight	Predicted weight	MNC/ml ($\times 10^6$)	CD34 ⁺ content (%)	Predicted CD34 ⁺ /kg ($\times 10^6$) Present weight	Predicted CD34 ⁺ /kg ($\times 10^6$) Future weight	% Target ^a
101	4.5	9.7	19	7.00	1.75	2.45	1.25	63 ^b
102	13	26	37	2.00	0.50	0.20	0.14	7
103	0.92	8	14	16.00	2.0	6.40	3.66	183
104	15.25	32	38	17.00	1.0	3.40	2.86	143
105	8.5	47.9	75	12.67	0.30	0.76	0.49	24
106	7.67	21	37	10.20	1.84	3.75	2.13	107
107	5.25	13.8	22	5.90	2.62	3.09	1.94	97
Mean	7.9	22.6	34.6	10.1	1.43	2.9	1.8	89.0
Std Dev	5.0	14.1	20.3	5.5	0.9	2.1	1.3	63

BM, bone marrow; FA, Fanconi anemia; G-CSF, granulocyte colony-stimulating factor; MNC, mononuclear cell. ^aTarget collection of 2×10^6 CD34⁺ cells/kg future weight. ^bRecommended G-CSF mobilization, family refused.

Table 3 Results of BM collection in FA patients

No. of patients	Pre-Hgb (g/dl)	Post-Hgb (g/dl)	Collected MNC/kg ($\times 10^8$)	CFUs/10 ⁵ MNC	CD34 ⁺ content (%)	CD34 ⁺ /kg ($\times 10^6$) Present weight	CD34 ⁺ /kg ($\times 10^6$) Future weight	% Target ^a
101	13.1	8	2.41	36	0.44	1.1	0.5	27
102 ^b	11.2	7.8	2.12	18	0.14	0.3	0.2	10
103	13.2	11.5 ^c	2.43	67	1.30	3.2	1.8	90
104	12.5	10.9 ^c	1.43	21	0.60	0.9	0.7	36
105 ^b	8.3	8 ^c	1.63	34	1.23	2.0	1.3	64
106	11.2	11.3 ^c	1.96	3	1.50	2.9	1.7	84
107	13.6	8.7	3.04	64	0.95	2.9	1.8	91
Mean	11.9	9.5	2.1	35	0.9	1.9	1.1	57
Std Dev	1.8	1.7	0.5	24	0.5	1.2	0.7	33

BM, bone marrow; CFU, colony-forming unit; FA, Fanconi anemia; G-CSF, granulocyte colony-stimulating factor; MNC, mononuclear cell. ^aTarget collection of 2×10^6 CD34⁺ cells/kg future weight. ^bReceived G-CSF mobilization before BM collection. ^cReceived a 10 ml/kg PRBC transfusion at the time of BM collection.

that did not meet these criteria or for which CD34⁺ content was unavailable at the time of selection were cryopreserved as a red blood cell-depleted mononuclear cell (MNC) preparation. CD34⁺ cell selection was also performed on a single cryopreserved red blood cell-depleted MNC preparation collected 5 years previously (FAAGT 1002). This cryopreserved collection was thawed for CD34⁺ cell selection specifically for participation in the gene transfer trial.

Post-selection CD34 purity ranged from 47 to 95% (Table 4), and recovery was good (48–133%) in three out of four of the fresh FA BM MNC products. Progenitor frequency was similar irrespective of cell source for the purified products, with the exception of the CD34⁺ cells purified from the previously cryopreserved FA BM. The pre-selection and post-selection CD34 frequencies were 1.2 and 78%, respectively, in this product and CD34⁺ recovery was low (40%), correlating with low

numbers of colony-forming units ($\sim 9/100$ CD34⁺ cells) in the CD34-selected population. This sample failed to expand in cytokine pre-stimulation conditions utilized for gene transfer (see below).

Gene transfer and re-infusion of transduced CD34 cells in FANCA (FA-A) patients

Three patients were entered into a phase I trial to assess the safety of gene transfer utilizing the MSCV-FANCA retrovirus vector. Patient demographics are shown in **Supplementary Table S1**. BM progenitor frequency ranged from ~ 6 to 18 per 10^5 MNC. Post-selection CD34 purity before pre-stimulation ranged from 47 to 78% (**Table 5**). After 36 h of pre-stimulation, cell yields of the two patients (FAAGT 1001 and 1003) from which freshly isolated CD34 cells were utilized were 95 and 99%, respectively. The cell yield of the product from FAAGT 1002 (cells derived from previously cryopreserved red cell-depleted MNC product—see above) was only 17%. After two separate transductions and a maximum of 84 h in culture, the final cell yields were 82 and 110% for FAGT 1001 and 1003 and 5% for FAGT 1002.

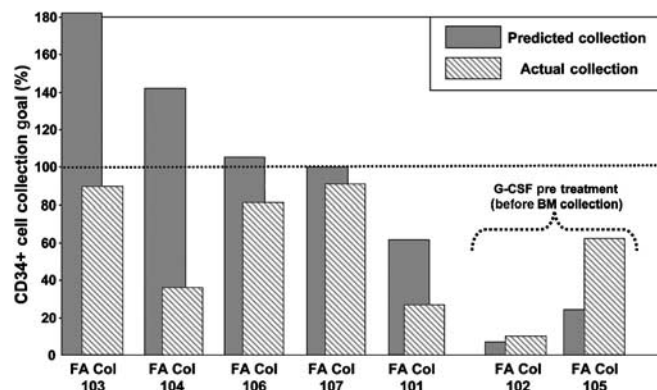


Figure 2 A comparison of predicted to actual CD34⁺ cell collection with a 20 ml/kg BM collection in FA patients.

Aliquots of transduced cells were plated in standard progenitor assays with or without exposure to 10 and 20 nM MMC, concentrations that effectively inhibit growth of non-corrected FA cells. As seen in **Table 5**, 40–48% of colony-forming units survived exposure to 10 nM MMC after gene transduction. At this concentration, there was no colony growth from non-transduced CD34⁺ cells from any patient. DNA isolated from individual FA-derived colony-forming units colonies from untreated progenitor assay plates demonstrated that 42–62% were polymerase chain reaction (PCR) positive for the presence of the MSCV-FANCA vector sequences. No PCR-positive colonies were detected from non-transduced specimens (33–36 colonies analyzed per patient).

Transduced cell products were evaluated for sterility and endotoxin levels and certified for re-infusion. Two patients (FAAGT 1001 and FAAGT 1003) were infused with transduced autologous products. Owing to the poor *in vitro* survival of the previously cryopreserved cells and the low numbers of cells after transduction, FAAGT 1002 did not receive a transduced product. The total nucleated cells/kg infused after transduction in FAAGT 1001 and 1003 were 4.5×10^5 and 3.5×10^5 , respectively. Re-infusions were tolerated without problems. Following infusion, blood was obtained weekly for 8 weeks and then monthly for the first year. BM was obtained at 3 months, 6 months, and 1 year. Provirus was not detected in PB or BM of FAAGT 1001 patient at any time. FAAGT 1003 showed 1–5 copies of the provirus/ 10^5 nucleated PB cells at 2 and 4 weeks post-infusion (**Supplementary Figure S1**). No provirus was detected in BM or marrow-derived progenitor colonies (30–36 colonies analyzed) at 3, 6, or 12 months. Replication competent retrovirus was not detected in any sample in either patient.

Both patients demonstrated a prolonged, although ultimately transient, increase in Hb and platelet counts (**Figure 3**). Despite no molecular evidence of gene-corrected cells *in vivo*, patient FAAGT 1001 had ~ 1 g increase in Hb for 10 months and a transient increase in platelet count. Patient FAAGT 1003 showed a more variable increase in Hb that peaked at 3 months post-infusion and an increase in platelet count (maximal at 1 month)

Table 4 CD34⁺ cell purification of fresh and previously cryopreserved RBC-depleted BM collections

Patient ID	Pre-CD34 selection				Post-CD34 selection					
	TNC (10^8)	CD34 ⁺ content (%)	CD34 ⁺ ($\times 10^6$)	Total CD34/kg ($\times 10^6$)	TNC (10^8)	CD34 ⁺ content (%)	CD34 ⁺ ($\times 10^6$)	Total CD34/kg ($\times 10^6$)	% CD34 ⁺ recovery	CFUs/ 10^3 CD34 ⁺ cell
FA Col 102	55	0.14	8	0.3	0.135	73	9.9	0.4	129	48
FA Col 103	19.4	1.31	25.4	3.2	0.15	82	12.3	1.5	48	27
FA Col 104	45.7	0.6	27.4	0.9	0.11	47	5.2	0.2	19	172
FA Col 107	42	1	42	3	0.59	95	56	4	133	31
FAAGT 1002 ^a	92.8	1.2	111	3.2	0.56	78	44	1.3	40	9

BM, bone marrow; CFU, colony-forming unit; FA, Fanconi anemia; MNC, mononuclear cell; RBC, red blood cell; TNC, total nucleated cell. ^aPreviously cryopreserved RBC-depleted BM MNC collection.

Table 5 Gene transfer of FANCA cDNA in FA-A patients

		FAAGT 1001		FAAGT 1003		FAAGT 1002			
Total nucleated cell dose per kg at pre-stimulation (% CD34 ⁺ cells)		5.4 × 10 ⁵ (73%)		3.2 × 10 ⁵ (47%)		1.0 × 10 ⁶ (78%)			
Total nucleated cells per kg post pre-stimulation (% of input)		5.1 × 10 ⁵ (95%)		3.17 × 10 ⁵ (99%)		1.7 × 10 ⁵ (17%)			
Total nucleated cells per kg post gene transfer (% of input)		4.5 × 10 ⁵ (82%)		3.5 × 10 ⁵ (110%)		0.5 × 10 ⁵ (5%)			
		Progenitor analysis ^a							
		Normal donor (N=3)		FAGT 1001		FAGT 1003		FAGT 1002	
		Pre	Post-GT	Pre	Post-GT	Pre	Post-GT	Pre	Post-GT
Functional correction	Real time PCR of CFU's								
0 nM MMC		113 ± 10	60 ± 25	35	12	81	33	7	17
10 nM MMC		79 ± 18 (70%)	45 ± 21 (74%)	0	5 (40%)	0	16 (48%)	0	8 (46%)
20 nM MMC		58 ± 13 (51%)	37 ± 22 (61%)	0	2.5 (20%)	0	12 (36%)	0	5 (29%)
Proviral marking^b		0	25 ± 4% (26/106)	0	42% (14/33)	0	40% (14/35)	0	62% (18/29)

^aPer 10³ cells plated; ^breal-time PCR of progenitor-derived colonies.

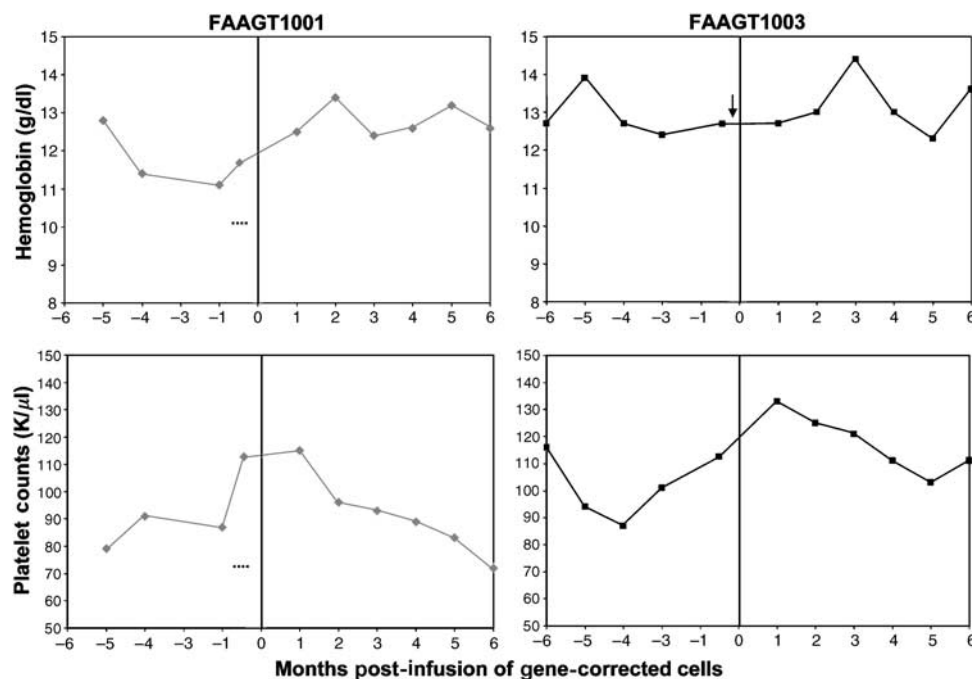


Figure 3 Peripheral Hb (top panels) and platelet counts (bottom panels) of FAAGT 1001 and FAAGT 1003 before and after re-infusion of their gene-corrected autologous hematopoietic cells. Horizontal interrupted line in FAAGT 1001 denotes G-CSF administration for mobilization. Arrowhead denotes red cell transfusion in FAAGT 1003.

that lasted >3 months, although the increase appears to have started before infusion of gene-corrected cells. There were no changes in the white blood cell counts (data not shown). There were no cytogenetic, fluorescence *in situ* hybridization, or morphologic changes in the BM for up to 1 year post-infusion in either patient (data not shown).

DISCUSSION

FA proteins have been postulated to be involved in multiple functions related to DNA repair, including detection of DNA

crosslinks, processing of DNA intermediates, recruitment of DNA repair proteins, or cell cycle checkpoint activation.²⁰ The exact role of FA proteins in these processes and their relationship to the progressive loss of stem cell function is unknown. In addition to the role of FA proteins in the nucleus, it has also been demonstrated that FANCA genes may have antiapoptotic functions²¹ and that FA hematopoietic progenitor cells are hypersensitive to proinflammatory cytokines,^{22,23} which are elevated in some FA patients.^{24,25} These observations may link the decline of HSC function over time owing to excessive apoptosis with the

evolution of oligoclonal hematopoiesis that is dependent on HSCs containing acquired secondary genetic mutations predisposing to leukemia. However, solid evidence for increased apoptosis in the HSC compartment of FA patients is lacking and this hypothesis remains unproven.

Based on this hypothesis, one would predict that small numbers of normal HSC will have a competitive advantage for repopulation in FA BM early in the disease before the evolution of HSCs bearing secondary genetic mutations. A competitive advantage of gene-corrected HSC/P has been demonstrated in murine models of Fancc deficiency,^{14,26} supporting the use of gene transfer in this disease, although the murine knockouts of FA genes are not phenocopies of the human disease with respect to progressive marrow aplasia and leukemia predisposition. Notably, some FA patients show evidence of acquired genetic reversion associated with improved hematopoietic function.¹⁵⁻¹⁷ The data from these rare patients have been interpreted to support the concept that corrected HSCs have a proliferative advantage *in vivo*. Given the limitations of the murine studies and the apparent heterogeneity in the cell of origin of the reversion events in humans, an unanswered question remains as to whether gene correction of FA HSC in humans will lead to a proliferative engraftment advantage *in vivo*. One previous gene transfer study in humans with FA did not resolve this issue.²⁷ Here we report the results of two related protocols established to address this question.

In these studies, we have focused on two key aspects of the FA phenotype. Many patients are referred to the Fanconi Anemia Comprehensive Care Center before significant cytopenias have developed; hence, we have attempted to collect and cryopreserve HSCs from these patients for future use. A major goal of the collection study was to determine whether clinically relevant numbers of HSC/P could be collected early in the disease. No previous studies have systematically addressed this issue in FA patients. Despite the small number of patients analyzed to date in the studies reported here, the following observations can be made. First, the pre-collection BM CD34 count overestimated the total CD34⁺ cell collection in FA patients who undergo a BM collection without prior treatment with G-CSF. No patient achieved the collection goal of 2×10^6 CD34⁺ cells/kg future weight in 5 years, although three of the four patients who met criteria for a BM collection achieved >80% of the collection goal. Second, by targeting the collection to 2×10^8 MNC/kg future weight in 5 years, rather than a maximum volume of 20 ml/kg, the collection goal would likely have been achieved in those patients with good cellularity. A maximum limit of 20 ml/kg BM harvest was chosen to limit the necessity of blood product support. However, in spite of this restriction, four patients required an erythrocyte transfusion at the time of BM collection, including three of the four patients with good BM cellularity. By removing this limit, the CD34⁺ cell collection goal would likely have been achieved in three out of four of these patients without increasing the number of patients requiring a blood transfusion. In the two patients who underwent a BM collection after failing to mobilize with G-CSF, targeting a cell dose rather than a maximum volume would have resulted in both patients

receiving blood products (instead of just one patient) but with the successful collection of 2×10^6 CD34⁺ cells/kg future weight in one patient. Our data suggest that FA patients have reduced CD34 content even before severe pancytopenia, a finding consistent with reduced hematopoietic progenitors found in single FA umbilical cord blood previously studied and reported.²⁸ It would appear that collection and cryopreservation of CD34⁺ cells at as young an age as possible is warranted in this disease. This study shows that such collections are feasible and well tolerated in FA patients.

Previous attempts by our group to collect G-CSF-mobilized HSC from FA patients also demonstrate mixed results.²⁹ These initial attempts to mobilize PB stem cells with G-CSF from patients with FA indicated that it may be feasible to collect adequate numbers of cells for retroviral infection and hematopoietic reconstitution. PB stem cells were collected from six patients with FA who achieved ≥ 6 CD34⁺ cells/mm³ after 6-14 days of 10 μ g/kg/day G-CSF stimulation. Two additional patients did not mobilize sufficient CD34⁺ cells to proceed. Six patients with FA who underwent apheresis mobilized a total of $6-16 \times 10^7$ CD34⁺ cells over 2-8 days of collection. Although the G-CSF mobilization time was prolonged in four patients (12-14 days) and collection times were greater than 3 days in two patients (7-8 days), all patients mobilized sufficient cells to demonstrate the feasibility of this approach. Although a majority of patients met collection targets in this previous study, this required prolonged G-CSF administration and extended numbers of apheresis, raising concerns about the quality of the product collected. Therefore, in this study, we attempted to use higher doses of G-CSF and limited days of treatment/apheresis without success. Newer mobilizing agents that target the CXCR-4 receptor³⁰ may prove useful in this regard and we are currently pursuing adding AMD3100³¹ treatment to G-CSF in an attempt to enhance mobilization of HSC/P in a select group of FA patients. Interestingly, the two patients receiving G-CSF were the only patients in the current collection trial in whom BM harvests met the CD34 target based on future needs. This result, although clearly preliminary, adds new human information to the reports of G-CSF "priming" in gene transfer studies in mice³² and non-human primates,³³ and suggests that G-CSF before BM harvest may be helpful.

A second goal of this study was to determine whether gene-corrected FA HSC/P could engraft in FA patients without the use of cytoreduction and, if engrafted, whether these corrected cells would show a proliferative advantage *in vivo*. This is an important question, as the success in gene transfer trials in X-severe combined immunodeficiency (SCID),^{7,34} ADA-SCID,⁸ and CGD⁹ all have in common either potent selective pressure for progeny of transduced progenitors (X-SCID and ADA-SCID) or the use of low doses of cytoreductive agents to enhance engraftment of *ex vivo* manipulated cells (ADA-SCID and CGD). Patients with FA have increased sensitivity to DNA-damaging agents, making the use of alkylators for cytoreduction unattractive. Although we utilized gene transfer methods that are considered state-of-the-art that were optimized for FA cells, and demonstrate excellent gene transfer efficiency in this clinical setting, we could not demonstrate either long-term

engraftment or selective advantage in this study. However, the lack of significant expansion of FA cells during *ex vivo* manipulation and/or defective engraftment of these cells after infusion into recipients likely contributed to the failure of gene-corrected cells to contribute to long-lived hematopoiesis. Indeed, it should be noted that the number of infused cells in this study is ~50-fold less than the successful studies noted above, in which up to 10×10^6 CD34⁺ cells/kg were infused into patients. Thus, one major conclusion from this study is that either more stem cells need to be collected for meaningful attempts at gene correction or improved conditions allowing maintenance or even enhanced engraftment of manipulated cells must be developed that specifically address the FA cellular phenotype. A second conclusion is that the methods utilized in this protocol appear to be safe and well tolerated to this point in follow-up.

Recent advances in understanding the biology of HSC engraftment offer some potential new approaches to the issue of poorly engrafting FA cells. These include the modulation of CD26,³⁵ HoxB4,³⁶ and RhoA³⁷ to enhance engraftment, the use of lentivirus vectors to significantly reduce the time required for *ex vivo* manipulations during HSC/P transduction, and even the complete elimination of *ex vivo* manipulations by direct intramedullary delivery of viral vectors. It is currently unknown whether any of the approaches will increase FA HSC/P gene transfer.

The transient increases in Hb and platelet counts seen in both patients in this study are worth noting for at least two reasons. First, without the molecular data demonstrating otherwise, interpretation of these changes as being related to gene correction would have been reasonable. Thus, careful interpretation of future results investigating gene transfer in this and other diseases is warranted. Second, one interpretation of these data is that exposure of primitive cells *in vitro* with the combination of cytokines utilized for “pre-stimulation” (or *in vivo* with high-dose G-CSF, GAGT 1001) contributed to an expansion of short-lived progenitor cells. Alternatively, “pseudotransduction”³⁸ of the FANCA cDNA could have provided short-term complementation, boosting cell production. These possibilities will be investigated further in animal models and in future patients.

Finally, in all cases, the issue of the presence or development of genomic mutations in the manipulated and gene-corrected cells and the relationship of these alterations to leukemia predisposition remains an important concern. However, given the results of allogeneic BM transplantation in FA patients without human leukocyte antigen-identical sibling donors, the development of alternative therapies in a selected group of FA patients is clearly warranted.

MATERIALS AND METHODS

All studies were approved by the Cincinnati Children's Hospital Medical Center's Institutional Review Board and the Institutional Biosafety Committee.

BM analysis from FA patients and normal volunteers. A BM aspirate (maximum 10 ml) was collected and CD34 content was measured according to the International Society of Hematology and Graft Engineering guidelines.³⁹ BM MNCs or purified CD34⁺ cell

populations (where indicated) were analyzed for progenitor content as described previously⁴⁰ and scored on day +14 after incubation in 5% pO₂ and 5% pCO₂ at 37°C at 100% humidity.

FA HSC collection. The collection protocol targeted $>2 \times 10^6$ CD34⁺ cells/kg (based on a predicted future weight in 5 years) either by a BM collection or through G-CSF-induced PB mobilization in FA patients. Patients without severe PB cytopenias could enroll in this study if they had no cytogenetic abnormalities or evidence of myelodysplastic syndrome or leukemia based on a BM aspirate performed within 3 months of collection. This BM analysis was utilized to calculate the volume of BM necessary to achieve the CD34⁺ cell collection goal. Based on marrow cellularity, we recommended marrow harvest (if the predicted CD34⁺ cell collection reached target numbers with a collection less than 20 ml/kg) or, as an alternative, high-dose G-CSF mobilization and collection. FA patients and families were informed of the relative risks and benefits of each procedure and allowed to choose between collection methods.

Mobilization was achieved with 16 µg/kg G-CSF (Amgen, Thousand Oaks, CA) given subcutaneously daily for 4 days. Daily peripheral leukocyte and CD34⁺ counts were obtained. Patients with ≥ 5 CD34⁺ cells/µl of PB were to undergo daily white blood cell apheresis (up to three total blood volumes) until the calculated collection target was achieved or for a maximum of 4 days. Patients with <5 CD34⁺ cells/mm³ of PB on day +5 of G-CSF were treated with increased G-CSF (16 µg/kg subcutaneously BID). A maximum of 8 days of G-CSF injections were allowed. Patients who failed to mobilize adequately or in whom apheresis failed to collect the target CD34⁺ cells after 4 days subsequently underwent a BM harvest (7–14 days after G-CSF injections).

Large-scale CD34⁺ cell selection. BM MNC preparations underwent CD34⁺ cell selection using the CliniMACS magnetic cell separator (CliniMACS, Miltenyi Biotec, Auburn, CA) according to the manufacturer's recommendations under a Food and Drug Administration Investigational New Drug application (BB IND No. 8828). Previously cryopreserved, erythrocyte-depleted BM MNC collections were thawed at 37°C and washed in dextran 40/4% human serum albumin solution containing 5 µg/ml Pulmozyme (Dornase alfa, Genentech, Vacaville, CA). The thawed BM MNC preparations were then resuspended in Miltenyi Selection Buffer solution containing 5 µg/ml Pulmozyme and subjected to CD34⁺ cell selection. The final product was sampled for viability, CD34⁺ cell and progenitor content, and sterility and then either cryopreserved for future autologous use or placed in culture for *ex vivo* stimulation and gene transfer.

Clinical FAA HSC gene transfer trial. A clinical gene transfer trial for FA patients with the FA-A complementation group was registered with the National Institutes of Health Recombinant DNA Advisory Committee and administered under a Food and Drug Administration Investigational New Drug application for the transduced CD34⁺ cells (BB IND8828). Eligibility criteria included FA-A patients, age >1 year, no evidence of leukemia or myelodysplastic syndrome, and $>1 \times 10^5$ CD34⁺ cells/kg available for *in vitro* gene correction. Individual patient entry and trial data were reviewed in real time by an independent external Data Safety and Monitoring Board.

Retroviral vector and transduction protocol. MSCV-FANCA is a murine stem cell virus-based retroviral vector⁴¹ containing the human FA-A cDNA under control of the retroviral long terminal repeat in the MSCV vector backbone. The MSCV-FANCA plasmid was transfected into the ecotropic GP+E86 retroviral packaging cell line and

supernatant was used to transduce the packaging cell line PG13⁴² at the National Gene Vector Laboratory at Indiana University. The PG13:MSCVFANCA GP-env-GALV-derived clone utilized for clinical scale production had the highest titer of multiple clones screened ($>1 \times 10^6$ infectious particles/ml, tested in HeLa cells).

Transduction of FA CD34⁺ selected cells was performed in serum-free medium (Stemline™ Hematopoietic Stem Cell Expansion Medium, Sigma-Aldrich, St Louis, MO) in the presence of 100 ng/ml stem cell factor and Flt-3 ligand (Flt3L) and 50 ng/ml interleukin-6 (IL-6) (PeproTech, Rocky Hill, NJ). Cells were placed in culture medium plus cytokines for pre-stimulation at a concentration of $1.5\text{--}3 \times 10^5$ cells/ml. After 36 h of pre-stimulation, cells were harvested, resuspended in a 1:1 mix of fresh medium/2 × cytokines and retroviral supernatant, and plated at a concentration of $2\text{--}5 \times 10^4$ cells/cm² ($2\text{--}5 \times 10^5$ cells/ml of transduction medium) on non-tissue culture flasks treated with Retronectin™ (Takara Bio, Otsu, Japan). The Retronectin-coated plates were preloaded twice with retroviral vector.^{43,44} After 4 h of exposure to the retroviral vector, an equal volume of fresh medium plus 2 × concentration of cytokines was added (final cell concentration of $1\text{--}2.5 \times 10^5$ cells/ml) and the cells were incubated overnight. The transduction step was repeated the next day. After a maximum of 84 h in culture, the transduced cells were harvested, washed, and prepared for re-infusion. Cells were only infused if the final cell infusion product (post-safety testing) had $>5 \times 10^4$ viable nucleated cells/kg.

Hematopoietic progenitor assays for FA gene transfer efficiency and functional correction. Gene transfer efficiency and functional correction studies were performed on normal and FA CD34⁺ cells after *ex vivo* gene transfer with the PG13:MSCVFANCA by standard hematopoietic progenitors assays. Post-transduction progenitors were grown in the absence or presence of MMC (10 and 20 nM) (Sigma-Aldrich) to assess functional correction. Individual colonies were isolated for DNA extraction and PCR analysis from both MMC-treated and untreated cells to confirm that functional correction was associated with the presence of the integrated provirus and to determine gene transfer efficiency.

PCR analysis. Quantitative PCR was used to detect the presence of provirus in individual progenitors (pre-infusion analysis and post-infusion monitoring) and MNCs were obtained from PB and BM samples. PB was obtained weekly for 8 weeks and then monthly as indicated. BM samples were obtained at 3 months, 6 months, and 1 year post-infusion of gene-corrected product. DNA was isolated using the Puragene Blood Kit and Puragene Cell and Tissue Kit (Gentra Systems, Minneapolis, MN). Quantitative PCR was performed using the ABI Prism 7700 sequencer detector (Taqman, PE Applied Biosystems, Foster City, CA). Primers and probes recognizing the MSCV-FANCA provirus contained the following sequences: forward primer, 5'-ACTGCGAGA GAGAGGAGCTATTG-3'; reverse primer, 5'-ACAAACGTGGAAAGCC TTTGG-3'; probe, 5'-(FAM)-CCTTGATGGCCTGCTCGTCAC (TAMRA)-3'. The human ApoB gene locus was used as an internal control. For progenitor samples, 5 μl of test material was added. For bulk DNA analysis, 1 μg of test material was added. The 25 μl reaction mix consisted of 300 nM of each FA primer, 80 nM of each ApoB primer, 200 nM of each probe, 1.2 mM dNTP, 5.5 mM MgCl₂, 0.05 units/μl AmpliTaq Gold, and 1 × TaqMan Buffer A. Amplification included one cycle of 50°C for 2 min, one cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were run in triplicate. Serial dilutions of single-copy HeLa clone with one proviral insert were run with each sample for copy number quantitation in bulk PB DNA.

Safety assessment and outcome. Before infusion of the transduced product, cells were collected for replication competent retrovirus testing

using the extended S⁺/L⁻ assay. After infusion, PB and BM from study participants were evaluated for retroviral GALV envelope sequence by PCR.⁴⁵ CBC with differential was performed at each time point along with periodic monitoring of renal and liver functions. BM was analyzed for cellularity and cytogenetics.

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SUPPLEMENTARY MATERIAL

Figure S1. Real time PCR detection of gene marked hematopoietic cells in FAAGT 1003's peripheral blood 3 weeks following re-infusion of gene corrected CD34⁺ cells.

Table S1. FA-A patients enrolled in HSC gene transfer study.

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