Lentiviral-mediated Genetic Correction of Hematopoietic and Mesenchymal Progenitor Cells From Fanconi Anemia Patients

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Previous clinical trials based on the genetic correction of purified CD34$^+$ cells with γ-retroviral vectors have demonstrated clinical efficacy in different monogenic diseases, including X-linked severe combined immunodeficiency, adenosine deaminase deficient severe combined immunodeficiency and chronic granulomatous disease. Similar protocols, however, failed to engraft Fanconi anemia (FA) patients with genetically corrected cells. In this study, we first aimed to correlate the hematological status of 27 FA patients with CD34$^+$ cell values determined in their bone marrow (BM). Strikingly, no correlation between these parameters was observed, although good correlations were obtained when numbers of colony-forming cells (CFCs) were considered. Based on these results, and because purified FA CD34$^+$ cells might have suboptimal repopulating properties, we investigated the possibility of genetically correcting unselected BM samples from FA patients. Our data show that the lentiviral transduction of unselected FA BM cells mediates an efficient phenotypic correction of hematopoietic progenitor cells and also of CD34$^-$ mesenchymal stromal cells (MSCs), with a reported role in hematopoietic engraftment. Our results suggest that gene therapy protocols appropriate for the treatment of different monogenic diseases may not be adequate for stem cell diseases like FA. We propose a new approach for the gene therapy of FA based on the rapid transduction of unselected hematopoietic grafts with lentiviral vectors (LVs).

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INTRODUCTION

Fanconi anemia (FA) is a rare chromosomal instability disorder characterized by congenital abnormalities, bone marrow (BM) failure and cancer predisposition, mainly acute myeloid leukemia and squamous cell carcinomas.[1] To date, 13 complementation groups have been identified, each associated with mutations in the corresponding FA gene.[2] Although allogeneic transplantation constitutes the preferential therapeutic option for FA patients, the output of patients transplanted with grafts from nonrelated donors is still modest.[3,4] Gene therapy is, therefore, considered a good therapeutic option for FA patients without human leukocyte antigen–identical siblings. Nevertheless, in contrast to results obtained in X-linked severe combined immunodeficiency,[5,6] adenosine deaminase deficient severe combined immunodeficiency,[7] or chronic granulomatous disease[8] patients, gene therapy trials conducted so far did not facilitate the engraftment of FA with autologous, genetically corrected cells.[9–11]

Taking into account that genetically corrected FA hematopoietic stem cells (HSCs)—either by natural processes[12,13] or after *ex vivo* gene therapy[14]—acquire a proliferation advantage *in vivo*, major aspects should account for the limited efficacy of gene therapy protocols already used in FA patients. In this respect, either the reduced number of transplanted CD34$^+$ cells or qualitative HSC defects associated to a disruption in the FA pathway[15,16] might account for the difficulties to engraft FA patients with corrected cells.[9–11] Additional aspects need, however, to be considered aiming to improve the efficacy of gene therapy in FA. First, although the repopulation potential of purified CD34$^+$ cells from X-linked severe combined immunodeficiency, adenosine deaminase deficient severe combined immunodeficiency, and chronic granulomatous disease patients is evident,[5–8] the question of whether purified CD34$^+$ cells from FA patients will be capable of engrafting these patients needs to be considered with caution, because FA is an HSC disease where the reproducible expression of the CD34 antigen in FA HSCs has not been demonstrated.

One additional aspect that may limit the engraftment of FA patients with genetically corrected cells relates to the sensitivity of FA HSCs to prolonged incubation protocols conventionally used for transduction with γ-retroviral vectors (RVs). In this respect,

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previous experimental studies with FA mouse models and also with samples from FA patients have already shown that FA progenitors/HSCs are particularly sensitive to *in vitro* manipulation due to the apoptotic and leukemic predisposition of these cells.[16–18] Although previous studies from our laboratory have shown the possibility of efficiently transducing hematopoietic progenitors from FA patients with RVs in short periods of time,[19] lentiviral vectors (LVs) nowadays constitute the most efficient vectors for stably transducing not prestimulated HSCs.[20] Moreover, previous studies in FA mouse models have already shown the efficacy of these vectors to genetically correct unselected FA HSCs in short periods of time,[21] and also demonstrated the relevance of conducting rapid transduction protocols to preserve the engrafting of transduced FA HSCs.[22]

In this study, we propose a rapid and clinically applicable lentiviral-mediated transduction procedure, capable of efficiently correcting the phenotype of total, not $CD34^+$ selected, BM cells from FA patients. Our data regarding the functional properties of FA $CD34^+$ cells, together with previous studies showing the engrafting facilitating properties of $CD34^-$ populations (see review in refs. 23 and 24), allow us to propose that the genetic correction of unselected grafts from FA patients should improve the efficacy of gene therapy in FA.

RESULTS

The content of $CD34^+$ cells in the BM of FA patients is not predictive of their hematological status

In order to evaluate the hematopoietic progenitors' content in the BM of FA patients, the proportion of $CD34^+$ cells and of colony-forming cells (CFCs) was determined in BM samples from 27 patients of the Spanish Registry of FA. Although a good correlation between values of $CD34^+$ and CFCs was initially expected, data in **Figure 1a** showed no correlation between these two parameters. With the aim of investigating whether either $CD34^+$ or CFC values were indicative of the hematological status of FA patients, both parameters were correlated with peripheral blood cell counts observed in each patient at the time of the BM aspiration. Although $CD34^+$ values did not correlate with any of the hematological parameters tested, good correlations were found between all tested hematological parameters and numbers of BM CFCs (**Figure 1b**). These results suggest that, compared to $CD34^+$ cell determinations, numbers of CFCs in the BM of FA patients may constitute a more reliable indicator of the hematopoietic function of these samples.

Based on our concerns about the functional properties of FA $CD34^+$ cells, and taking into account previous observations showing that the infusion of accessory $CD34^-$ populations facilitated the engraftment of donor cells in transplanted recipients,[23] we explored the possibility of genetically correcting FA BM samples not subjected to any immunoselection step; a process that may additionally reduce the number of FA HSCs.

Efficient lentiviral transduction and phenotypic correction of unselected BM progenitors from FA-A patients

Because Fanconi anemia group A (FA-A) is the most frequent complementation group in FA patients,[25,26] LVs expressing the *FANCA* gene and/or the enhanced green fluorescent protein (*EGFP*) marker gene were used throughout (see Materials and Methods). These LVs were pseudotyped with a chimeric glycoprotein from the GALV and the MLV-A viruses (GALV-TR[27]) based on previous studies from our laboratory showing the efficacy of GALV-packaged RVs to transduce FA progenitor cells.[19]

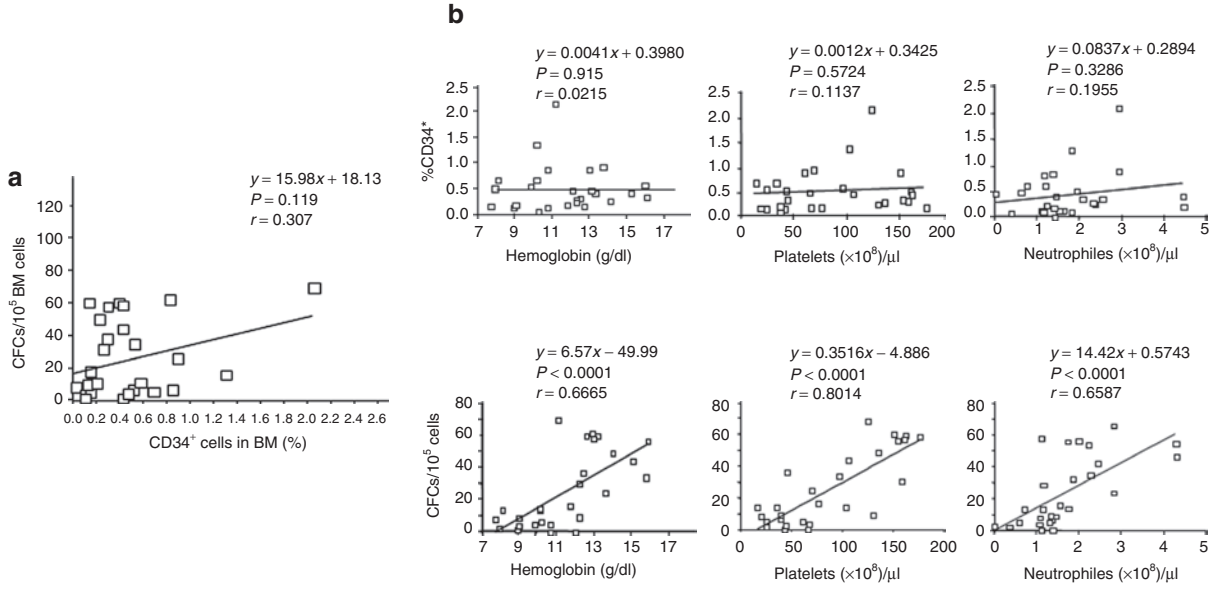

Figure 1 Functional significance of BM $CD34^+$ cells and CFCs in FA patients. (**a**) Correlation analysis between the percentage of $CD34^+$ cells and the number of CFCs/10^5 cells in the BM of FA patients. Percentages of $CD34^+$ cells and numbers of CFCs/10^5 cells in the bone marrow of healthy donors were 1.6 ± 0.2% and 191.3 ± 37.9, respectively. (**b**) Correlation between either the percentage of bone marrow $CD34^+$ cells or the proportion of bone marrow CFCs with respect to relevant hematological parameters of these patients. BM, bone marrow; FA, Fanconi anemia; CFC, colony-forming cell.

In a first set of experiments, Ficoll-Paque (F-P) fractionated BM samples were transduced in plates preloaded with the LVs (see static preloading protocol in **Supplementary Figure S1a**). As shown in **Table 1**, transduced samples contained an average of 70% of the input cellularity and 120% of CFCs numbers determined before the transduction process, indicating modest variations throughout the transduction period. The transduction efficacy of the protocol determined in the CFCs was around 50%. Transductions with LVs coexpressing the *FANCA* and the *EGFP* genes mediated CFCs survivals to mitomycin-C that correlated with the proportion of EGFP-expressing colonies (**Supplementary Figure S2**). Because the hypersensitivity to DNA crosslinking agents is a hallmark of FA cells, these results show the efficient phenotypic reversion conferred by FANCA-expressing LVs on FA progenitor cells and evidences that transductions with *FANCA*/*EGFP* LVs can be determined by analyses of either green fluorescent colonies or mitomycin-C-resistant colonies (colonies resistant to 10 nmol/l mitomycin-C).

The cryopreservation of FA BM impairs the efficacy of the transduction protocol

Although in several monogenic diseases, genetically corrected cells can be infused to the patient a few days after the harvesting of the graft, in FA it might be convenient to space the harvesting and the infusion of the cells further apart (see Discussion). In these cases, it would be necessary to transduce cryopreserved, rather than fresh samples, for gene therapy purposes. To investigate the possibility of efficiently transducing unselected cryopreserved BM cells from FA patients, samples were stored in liquid nitrogen, and then thawed and transduced as in experiments of **Table 1**. Historical data from our laboratory showed that on average 70% of cryopreserved FA-A CFCs were recovered after the thawing process ($n = 18$; data not shown). Similar recoveries were obtained in experiments aiming the transduction of cryopreserved cells (**Table 2**). These experiments also showed that during the 16-h transduction process, the number of CFCs further decreased about 50% (**Table 2**), which markedly contrasts to data obtained with fresh samples where CFCs numbers slightly increased during the transduction period (**Table 1**). Transduction efficacies of cryopreserved CFCs were also significantly lower compared to efficacies obtained in fresh CFCs (24 vs. 50% of CFCs transduction, respectively). This observation was confirmed in BM samples that were transduced with the same LV production batch, before and after cryopreservation (*i.e.*, see patients FA170, and FA328 in **Tables 1** and **2**). Incubations of thawed cells for up to 24 hours at 37 °C did not improve the transduction efficacy of cryopreserved BM samples (**Table 2**).

Optimized transduction procedures are required for the efficient transduction of cryopreserved BM samples from FA patients

Based on the difficulties to transduce cryopreserved FA BM progenitors, further studies were conducted aiming to increase the total number of HSCs that could be subjected to the gene transfer protocol. In this respect, although fractionation with F-P constitutes a routine procedure for the preparation of BM mononuclear cells, data obtained from a total of 32 FA BM samples (including those presented in **Tables 1** and **2**) showed that the recovery of $CD34^+$ cells was very low (mean value: 51%), compared to recoveries achieved with healthy BM samples (87% on average). Although we have shown in **Figure 1** that $CD34^+$ cell numbers may not represent the functional properties of FA BM samples, our results in **Tables 1** and **2** suggest the convenience of using a fractionation method alternative to F-P, in order to optimizing the number of HSCs that can be transduced by the therapeutic vectors. Because erythrocyte sedimentation with hydroxyethyl starch (HES) constitutes a routine procedure for the preparation of cord blood units

Table 1 Characteristics of fresh bone marrow samples from Fanconi anemia patients transduced with lentiviral vectors expressing EGFP and/or FANCA

		Lentiviral vector		Fresh BM properties	Transduced bone marrow				
					Recovery (% pretransduction)		Properties of transduced sample		
Exp.	Patient	Gene	Titer (×10^5)	CFCs/10^5 cells	Cells	CFCs	CFCs/10^5 cells	% $EGFP^+$	Survival to MMC (%)
E1	FA164-2 (A)	*EGFP*	2.0	5.0	46.2	207.1	22.4	50.0	0
E2	FA342 (NS)		2.0	35.7	71.0	159.6	80.3	45.0	0
E3	FA330 (A)		2.0	4.6	40.9	66.6	7.5	32.1	0
E4	FA328 (A)		6.5	12.0	86.0	73.1	10.2	62.4	0
E5	FA170 (A)		6.5	89.3	90.3	43.8	43.3	64.2	11.1
Mean ± s.e.			3.8 ± 1.1	29.3 ± 16.0	66.9 ± 10.1	110.0 ± 31.2	32.7 ± 13.5	50.7 ± 5.9	2.2 ± 2.2
E1	FA164-2 (A)	*FANCA/EGFP*	2.4	5.0	57	205.1	18.0	58.0	62.5
E2	FA342 (NS)		2.4	35.7	74.2	198.2	95.4	14.2	10.3
E3	FA330 (A)		2.4	4.6	50.5	102.1	9.3	31.4	68.8
E4	FA328 (A)		5.5	12.0	89.2	92.9	12.5	67.3	57.6
E5	FA170 (A)		5.5	89.3	92.5	33.1	32.0	58.3	68.8
Mean ± s.e.			3.6 ± 0.6	29.3 ± 13.3	72.7 ± 7.1	126.3 ± 27.9	33.4 ± 13.5	45.8 ± 8.4	53.6 ± 11.0

Bone marrow cells were fractionated with Ficoll-Paque and then transduced for 16 hours according to the static lentiviral preloading method shown in **Supplementary Figure S1a**. (A), FA-A complementation group; (NS), not subtyped; $EGFP^+$, % of green fluorescent colonies; concentration of MMC, 10 nmol/l. EGFP, enhanced green fluorescent protein; FA-A, Fanconi anemia group A; MMC, mitomycin-C.

Table 2 Characteristics of cryopreserved bone marrow progenitors from Fanconi anemia patients transduced with lentiviral vectors expressing EGFP and/or FANCA

				Cryopreserved and thawed BM			Transduced bone marrow					
		Lentiviral vector		Recovery (% prefreezing)		Properties		Recovery (% pretransduction)		Properties		
Exp.	Patient	LV	Titer (×10^5)	Cells	CFCs	CFCs/10^5 cells	Incubation period[a] (h)	Cells	CFCs	CFCs/10^5 cells	% EGFP$^+$	Survival to MMC (%)
E6	FA23 (A)	*EGFP*	6.6	115.3	97.0	37.1	—	57	58.4	37.9	23.5	0
E7	FA170 (A)		6.5	75.3	57.7	68.4	—	45.2	22.9	34.6	23.0	3.2
Mean ± s.e.			6.5 ± 0.0	95.3 ± 16.3	77.3 ± 16.0	52.7 ± 13		51.1 ± 4.8	40.6 ± 14.5	36.3 ± 1.3	23.2 ± 0.3	1.6 ± 1.3
E6	FA23 (A)	*FANCA/EGFP*	6.6	115.3	97.0	37.1	2	60.2	58.0	38.4	30.0	27.2
E7	FA170 (A)		5.5	75.3	57.7	68.4	2	51.6	46.6	61.8	19.2	11.5
E8	FA328 (A)		5.5	68.8	24.1	17.4	2	36.1	37.2	17.9	30.0	ND
							4	36.1	35.3	17.0	19.3	ND
							8	37.4	35.7	16.6	25.8	ND
E9	FA342 (NS)		1.8	52.5	100.8	68.8	2	31.2	45.2	99.7	23.3	ND
							4	25.8	53.4	142.5	31.6	ND
							8	36.6	54.4	102.4	24.7	ND
							24	11.8	40.9	238.0	26.5	ND
E10	FA330 (A)		1.0	101.7	48.3	5.4	2	46.2	52	6.1	ND	23.0
Mean ± s.e.			4.1 ± 1.0	82.7 ± 11.4	65.7 ± 14.7	40.3 ± 10.6		35.7 ± 4.2	45.9 ± 2.7	70.6 ± 19.6	25.4 ± 1.4	20.6 ± 3.5

Bone marrow cells were fractionated with Ficoll-Paque and then transduced for 16 hours a according to the static lentiviral preloading method shown in **Supplementary Figure S1a**. (A), FA-A complementation group; (NS), not subtyped; ND, not done; EGFP$^+$, % of green fluorescent colonies; concentration of MMC, 10 nmol/l.
[a]Samples were incubated at 37 °C prior to expose the samples to lentiviral vectors.
BM, bone marrow; CFC, colony-forming cells; EGFP, enhanced green fluorescent protein; FA-A, Fanconi anemia group A; LV, lentiviral vector; MMC, mitomycin-C.

and also for the purification of FA CD34$^+$ cells in previous clinical trials,[11] we investigated the efficacy of this very simple procedure to prepare a white BM cell population, free from erythrocytes that could interfere with the transduction of the sample. In contrast to the poor efficacy of F-P fractionation, almost all CD34$^+$ cells present in FA BM samples were recovered after a single gravity sedimentation of erythrocytes in HES (**Supplementary Table S1**).

With the purpose of improving the transduction of cryopreserved FA precursors, a new set of experiments was conducted, first in FA lymphoblast cell lines (FA-LCLs) and thereafter in cryopreserved FA BM samples. Although repetitive preloadings with infective vectors are frequently used to improve the transduction of target cells, our data in FA-LCLs show that one single preloading of GALV-TR packaged LVs is as efficient as four repetitive preloadings, regardless of the infectivity of the supernatant (**Figure 2a**). In further experiments, we observed that the centrifugation of LV-supernatants during the preloading process (dynamic preloading in **Supplementary Figure S1b**) increased by twofold the transduction rate of LCLs (**Figure 2b**). Additionally, compared to preloadings conducted at 4 °C, preloadings at temperatures of 22 and 32 °C progressively increased the transduction efficacy of the LCLs (**Figure 2c**). Finally, in five different experiments with FA-LCLs we demonstrated that repetitive transduction cycles (each transduction cycle was conducted in plates treated by one dynamic LV preloading) further improved the transduction of LCLs (**Figure 2d**). In these studies, we observed that the optimized volume of infective supernatant was of 0.2 ml/cm^2 (not shown).

In a next set of experiments, we aimed to confirm the efficacy of transduction obtained in LCLs using cryopreserved FA BM samples. As we observed in FA-LCLs, three transduction cycles significantly increased the transduction efficacy of FA CFCs compared to values obtained after a single transduction cycle (45.7 ± 4.2 versus 13.5 ± 5.1%, respectively; **Figure 3a**). Improved transductions were evident both in F-P and HES fractionated FA samples (**Supplementary Table S2**). This table additionally shows that similar yields of CFCs were obtained when samples were transduced with one or three infection cycles (total transduction period: 16 hours in both instances).

Finally, we investigated the relevance of maintaining FA BM cells at low oxygen concentration during the transduction process. In these studies, cryopreserved samples were subjected to three cycles of transduction, either at 21 or 3% oxygen concentration. Colonies generated after each transduction condition were grown both under high and low oxygen concentrations. As shown in **Figure 3b**, similar colony numbers (CFCs) and transduction efficacies were obtained in samples transduced under high and low oxygen concentration. Nevertheless, CFC's numbers were always higher when colonies were grown at 3% O_2. This final observation suggests the convenience of manipulating these samples under low oxygen concentration, even during the short incubations (16 hours) required for the transduction with the LVs.

The lentiviral transduction of unselected BM from FA patients facilitates the genetic correction of accessory mesenchymal stromal cells

With the final purpose of investigating whether our transduction conditions facilitated the transduction of CD34$^-$ mesenchymal

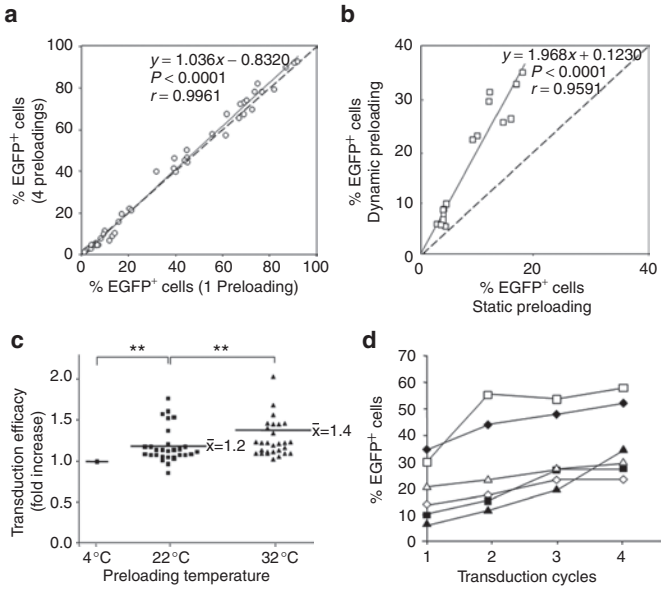

Figure 2 Improved transduction of Fanconi anemia lymphoblast cell lines mediated by dynamic preloadings of GALV-TR packaged lentiviral vectors. (**a**) Similar transduction efficacies mediated by one or four static preloadings of infective LVs in human FA-A lymphoblasts. (**b**) Improved transduction efficacy mediated by the dynamic preloading of infective supernatants (data show transduction efficacies induced by a single preloading with the vectors). (**c**) Relative improvement in the infectivity of LVs when dynamic preloadings were conducted at 4, 20, and 32°C. $^{**}P < 0.05$. (**d**) Improved infectivity associated to repetitive transduction cycles, each consisting of a single dynamic preloading of 2 hours followed by a transduction of 4 hours. Data corresponding to six individual experiments are shown. In all instances samples were transduced with EGFP-LVs. Analyses of $EGFP^+$ cells were made 5 days after transduction by flow cytometry. Each point represents transduction efficacies conferred by supernatants with a wide range of infectivity. EGFP, enhanced green fluorescent protein; FA-A, Fanconi anemia group A; GALV-TR, gibbon ape leukemia virus-terminal repeat, LV, lentiviral vector.

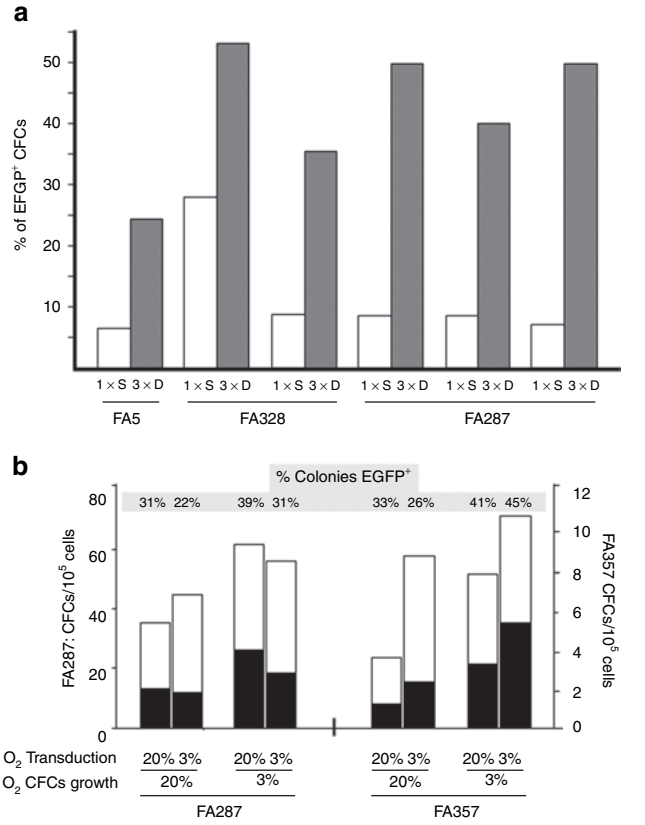

Figure 3 Improved transduction efficacy of cryopreserved BM progenitors from FA patients. (**a**) Samples were subjected to standard transductions consisting in a single transduction cycle (16 hours) after 2 hours of static preloading (white bars; 1 × S) or improved transduction consisting in three transduction cycles (2 hours + 2 hours + 12 hours) each mediated by a dynamic preloading (2 hours) of the lentiviral vectors (grey bars; 3 × D). (**b**) Analysis of the relevance of transducing FA BM samples (three cycles of transduction as in panel **a**) and culturing the hematopoietic progenitors (14 days of CFCs growth) in low (3%) or conventional (21%) oxygen concentration. White bars represent CFCs/10^5 cells and black bars the % of transduction. BM, bone marrow; CFC, colony-forming cell; FA, Fanconi anemia.

stromal cells (MSCs), aliquots of unselected BM cells were transduced under optimized conditions (**Supplementary Figure S1b**), and then cultured to facilitate the expansion of the MSCs. After three passages in culture an almost pure population of MSCs was obtained. As shown in **Figure 4a**, the expression of *EGFP* marker gene was confirmed in expanded MSCs, which displayed a characteristic phenotype consisting of $CD45^-$, $CD34^-$, $CD117^-$, $CD29^+$, $CD73^+$, $CD90^+$, $CD166^+$, and $CD44^+$. The pluripotential differentiation ability of these cells was confirmed by determining their capacity to differentiate into osteogenic and adipogenic lineages (**Figure 4b**). The phenotypic correction of FA-A MSCs in cells transduced with *FANCA/EGFP* LVs was investigated by analyzing the presence of FANCD2 foci in the nucleus of green fluorescent MSCs, because only gene complemented FA-A cells can generate FANCD2 foci (reviewed in ref. 2). As shown in **Figure 4c**, BM samples originally transduced with *FANCA*-expressing LVs—but not with control EGFP-LVs—generated MSCs with evident FANCD2 foci, demonstrating the restored functional FA pathway in these cells. As far as we know, this is the first description of a gene therapy approach that facilitates the genetic correction of hematopoietic progenitors and also of accessory cells like the MSCs.

DISCUSSION

Although gene therapy constitutes a promising alternative for the treatment of FA patients, none of the gene therapy trials conducted so far has facilitated the stable engraftment of these patients with genetically corrected cells.[9–11] Taking into account that FA gene therapy trials were similar to protocols employed for the genetic treatment of severe combined immunodeficiency and chronic granulomatous disease patients,[5–8] specific problems related to the physiopathology of FA should account for the current inefficacy of gene therapy in this genetic instabilty syndrome. In particular, in contrast to other monogenic diseases where functional defects only take place in maturing or functionally mature cells, mutations in FA genes result in functional defects in all cells, including the HSCs.[16,19,28,29] This observation indicates that specific manipulation procedures should be developed for the genetic treatment of FA HSCs.

Although all previous trials aiming the genetic correction of hematopoietic diseases used purified $CD34^+$ cells as targets of the therapeutic vector, it is currently unknown whether in the case of

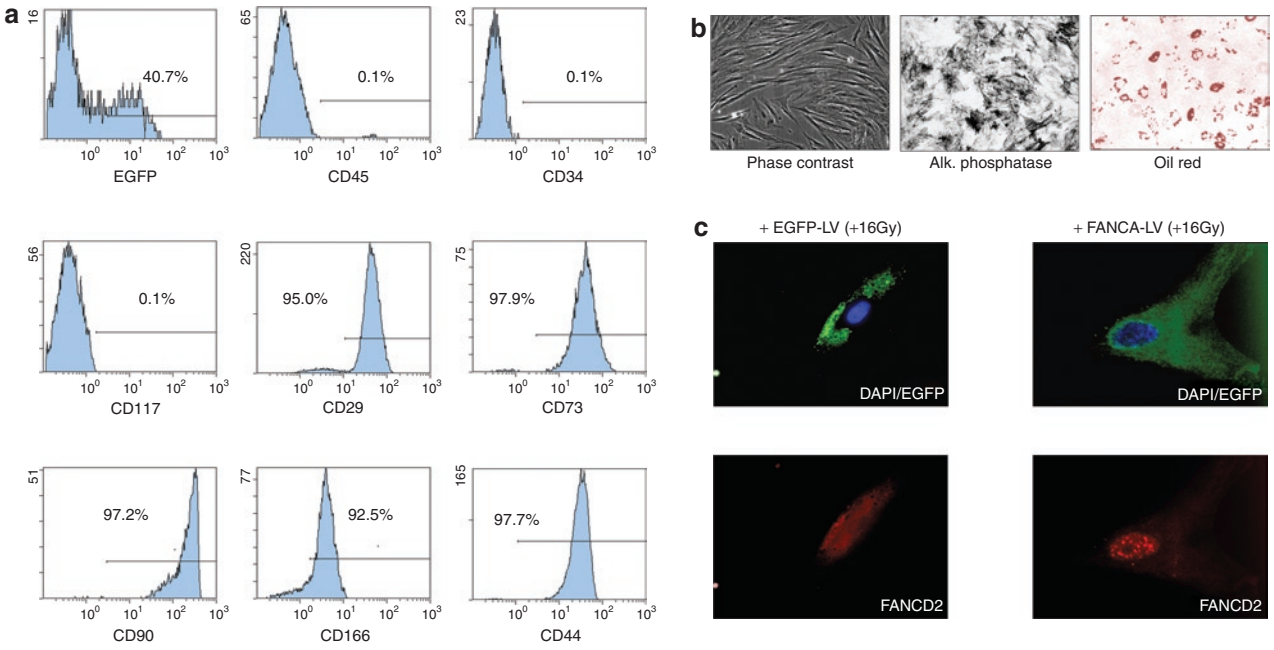

Figure 4 Generation of lentivirally transduced stromal stem cells from unselected Fanconi anemia bone marrow samples transduced under optimized conditions. (**a**) Characteristic phenotype of MSCs generated by unselected FA bone marrow samples transduced with LVs expressing the *FANCA* and *EGFP* transgenes, under conditions shown in **Supplementary Figure S1b**. (**b**) Phase-contrast microphotography of MSCs three passages after transduction with LVs and differentiation of MSCs into osteogenic (phosphatase alkaline positive cells) and adipogenic lineages (oil red positive cells), respectively. (**c**) Restored generation of FANCD2 foci in MSCs generated by BM previously transduced with *FANCA*/*EGFP* LVs. As a control, a sample transduced with a control LV (EGFP-LV) is shown. In all instances FANCD2 foci were determined in green fluorescent cells. Alk. phophatase, alkaline phosphatase; MSC, mesenchymal stromal cell; LV, lentiviral vector.

stem cell diseases like FA, purified $CD34^+$ cells are actually able to repopulate the hematopoiesis of FA patients. In this study, we first observed the absence of a correlation between $CD34^+$ cell values and CFCs values determined in the BM of FA patients. This is in marked contrast to the good correlations generally observed in hematopoietic samples from healthy donors[30,31] or cancer patients.[32,33] More interestingly, our data show that only determinations of BM CFCs, but not of $CD34^+$ cells, correlated with the hematological status of the patients. These results suggest that in the particular case of FA, the expression of the $CD34^+$ antigen may not constitute an adequate marker to characterize, and therefore to purify, BM precursors with *in vitro* and/or *in vivo* hematopoietic function. However, technical limitations to engraft immunodeficient mice with human FA hematopoietic cells do not allow us to demonstrate whether these observations imply a defect of FA $CD34^+$ cells to engraft, either in the short- or the long-term, transplanted recipients.

Apart from our concerns about the functional properties of FA $CD34^+$ cells, previous data suggest that the transduction of unselected FA grafts may improve the engraftment of genetically corrected cells. In this respect, it is interesting to note that previous studies have shown that normal $CD34^-$ cells contain engraftment-facilitating cells capable of improving the engraftment of immunodeficient mice with human hematopoietic cells.[24] Consistent with this observation, more recent studies have established a role of $CD34^-$ MSCs in hematopoietic engraftment, mainly after transplantation of reduced numbers of HSCs (see review in ref. 23). In FA, the re-infusion of transduced unselected BM cells, containing both $CD34^+$ and $CD34^-$ cells, might be particularly relevant to facilitate the engraftment of the patients, either because of the low number of autologous HSCs available for transplantation, or because of qualitative defects in the FA HSCs.

Aiming to optimize the transduction conditions of FA HSCs, recent studies with *Fanca*$^{-/-}$ mice have shown the relevance of conducting rapid transductions of FA samples with LVs in the engraftment of recipient mice with genetically corrected HSCs.[22] This observation together with data showing that LVs currently constitute one of the most efficient[34,35] and safe vectors[36,37] for stably transducing HSCs in short periods of time suggests the convenience of using LVs, rather than RVs for the gene therapy of FA. Due to the efficacy of GALV-packaged RVs to transduce BM progenitors from FA patients,[19] in the current studies LVs were packaged with a GALV envelope modified for the packaging of LVs (GALV-TR).[27] This envelope allowed us to transduce FA cells with LVs preloaded into retronectin-coated plates; a procedure that improves the transduction of the target cells[38] and limits the toxicity of the infective supernatant.[39,40] Additionally, the packaging of the vectors with these envelopes would prevent potential fusogenic effects of the vesicular stomatitis virus-G protein[41] on the very sensitive FA cells, and also facilitate the production of large batches of not concentrated infective GALV-TR packaged LVs. Our data in **Table 1** show that an overnight transduction of unselected FA BM cells under the above-mentioned conditions mediates an efficient phenotypic reversion of FA CFCs. Remarkably, these results were obtained after transductions with moderate titers of nonconcentrated LVs (titers around 5×10^5 transforming units/ml). These titers, and consequently the multiplicity of infections used in our studies, were markedly lower (between 10- and 100-fold lower) compared to multiplicity

of infections conventionally used for the transduction of samples with vesicular stomatitis virus-G packaged LVs,[22,42,43] thus limiting potential toxic effects mediated by an excess of LV particles.

In many inherited diseases like X-linked severe combined immunodeficiency, adenosine deaminase deficient severe combined immunodeficiency, or chronic granulomatous disease, hematopoietic autologous grafts can be transduced and re-infused in the patient immediately after the harvesting of the sample.[5–8] In contrast to these monogenic diseases, in FA patients the HSCs progressively die or suffer transformation. Therefore, in these patients hematopoietic grafts should be ideally collected during the early stages of the disease, although the infusion of genetically corrected cells may be recommended later on, once the hematological manifestation of the disease is apparent. In these cases the genetic treatment should be conducted on cryopreserved grafts harvested during the early stages of disease. Our data in **Tables 1** and **2** allow us to make a comparative study on the susceptibility of fresh and cryopreserved FA CFCs to LVs. Our results show that the LV-transduction of cryopreserved FA BM cells is associated with a significant mortality and a reduced transduction rate of the CFCs, compared to results obtained with fresh FA samples. These observations were not reproduced when cryopreserved BM cells from healthy donors were used (data not shown). Our studies also show that results were not improved when cryopreserved samples were preincubated for up to 24 hours before the transduction process. Longer incubation periods were not tested because prolonged *ex vivo* treatments of these samples may result in detrimental effects, as already shown in mouse models.[18,22]

Although cryopreservation of FA cells previously subjected to genetic correction might constitute a good alternative to the transduction of cryopreserved FA cells, this approach would imply the genetic correction of all BM grafts considered for cryopreservation. Because presumably only a proportion of FA patients will be finally treated by gene therapy, we consider that this strategy may not be practical in terms of a gene therapy trial. Additionally, this approach would limit the possibility of transducing FA cryopreserved grafts with improved vectors that might be developed in the future. Based on our observations, and given the low number of hematopoietic progenitors and HSCs present in the hematopoietic tissues of FA patients,[11,19,29,44] we reasoned the necessity of further improving the different *ex vivo* manipulation steps required for transduction of cryopreserved FA BM samples.

Although F-P fractionation is a routine procedure for the separation of mononuclear cells, we observed that a very simple sedimentation in HES constitutes a much more efficient and gentle procedure for the preparation of FA BM cells to LV-transduction. Our results also show that conducting three cycles of transduction on plates preloaded with LVs under low-speed centrifugation markedly increases the transduction efficacy of cryopreserved FA CFCs (46% on average; fourfold increase compared to standard transductions). Transductions conducted under low and high oxygen concentration showed no significant differences on the efficacy of transduction of FA CFCs. Nevertheless, the high number of colonies obtained when clonogenic assays were conducted at 3% O_2, and the convenience of limiting the oxidative damage on the very sensitivity of FA cells,[45] indicates that FA grafts should be maintained under low oxygen concentration, even during the short period required for the LV-transduction.

Due to current limitations to engraft immunodeficient animals with BM from FA patients, this study fails to demonstrate whether our transduction conditions facilitated the transduction of human FA HSCs. Nevertheless, observations in animal models[21,22,42,43] and also in human patients[46] have already shown that short-exposures to LVs (1–20 hours) can efficiently transduce long-term repopulating cells, either purified or not,[21] strongly suggesting that our transduction procedure will also efficiently transduce the FA HSCs.

In addition to the efficacy of our approach to transduce precursor cells of the hematopoietic lineage, here we show that the LV-transduction of unselected FA BM cells mediates the transduction of accessory MSCs. Moreover, the presence of nuclear FANCD2 foci in MSCs generated from unselected samples previously transduced with *FANCA*/*EGFP*-LVs, demonstrates the phenotypic reversion of these cells. This observation, together with previous data showing the role of $CD34^-$ cells,[24] including expanded or not expanded MSCs,[23] in hematopoietic engraftment, strongly suggests that the transduction of unselected FA BM cells with therapeutic LVs, may facilitate the engraftment of FA patients with genetically corrected cells.

Finally, although the feasibility of transducing unselected BM grafts in the context of a gene therapy trial might be questioned, it should be considered that most eligible FA patients will be pediatric patients with a reduced BM cellularity. Considering conventional parameters of BM harvesting we estimate, for a 5-year-old patient, a total number of 1.3×10^9 BM cells for transduction. The production of LV-supernatants with a titer of 10^6 transforming units/ml would imply the use of about 1.3 l of infective supernatant per transduction cycle. This volume is similar to the RV supernatant volume currently used in our laboratory for the genetic treatment of the graft-versus-host disease, where unselected peripheral blood T cells are transduced with HSV-*tk* RVs.[47]

Taken together, our results suggest that gene therapy protocols appropriate for the treatment of different monogenic diseases may not be adequate for stem cell diseases like FA, where all the hematopoietic, and also nonhematopoietic cells residing in the BM are affected by the genetic defect. The proposed genetic treatment of unselected FA BM grafts with LVs stably expressing FA genes (A. González-Murillo, M.L. Lozano, L. Alvarez, A. Almarza, S. Navarro, G. Guenechea *et al.*, unpublished results) may constitute an important step to improve the clinical efficacy of gene therapy in FA.

MATERIALS AND METHODS

Cell lines. 293T and HT1080 cells (CRL-12103 ATCC number; ATCC, Rockville, MD) were used for the production and titration of the LVs, respectively. These cell lines were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Biowhitaker, Europe, Belgium). Epstein–Barr virus–transformed LCLs were grown in Roswell Park Memorial Institute medium (Gibco) supplemented with 20% fetal bovine serum and antibiotics (0.5% of penicillin and streptomycin).

Lentiviral vectors. LV carrying the EGFP under the control of the internal spleen focus forming virus (SFFV) U3 promoter (SFFV-EGFP LV)

was used as control vector. As a therapeutic vector, an LV carrying the *hFANCA*IRES*EGFP* cassette was constructed (SFFV-hFANCA-I-EG; Gonzalez-Murillo *et al.*, unpublished results). Vector stocks were prepared by four-plasmid calcium–phosphate mediated transfection in 293T cells, as previously described,[48] using the GALV-TR envelope[27] (kind gift of F.L. Cosset). Supernatants were recovered 24 and 48 hours after transfection and filtered through 0.45 µm. Functional titers of infective LVs were determined in HT1080 cells, plated at 3.5×10^4 cells per well in 24-well plates and infected overnight with different dilutions of either LV-supernatants. Cells were washed and incubated with fresh medium, and the proportion of $EGFP^+$ cells was determined 5 days later by flow cytometry, as described.[48]

Patients and BM analyses. Studies were approved by the authors' Institutional Review Board and conducted under the Declaration of Helsinki. Patients were encoded to protect their confidentiality, and written informed consent obtained in all cases. FA patients were diagnosed on the basis of clinical symptoms and chromosome breakage tests of peripheral blood cells using a DNA crosslinker drug. The percentage of $CD34^+$ cells in fresh BM was determined according to previously standardized procedures.[49] For the assessment of CFCs, samples consisting of at least 300,000 mononuclear or erythrocyte-depleted BM cells were cultured in triplicates, in Methocult H4434 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with etanercept (anti-tumor necrosis factor-α fusion protein; 10 µg/ml final concentration; Immunex, Seattle, WA) at 37 °C, in 5% CO_2, and 95% humidified air, according to standardized procedures.[19] Where indicated, cultures were incubated under the same conditions, but with a low oxygen concentration (3% O_2). Colonies were scored after 2 weeks in culture.

Fractionation of BM cells with Ficoll-hypaque or HES. Mononuclear bone marrow cells were obtained by fractionation in F-P, according to manufacturer's instructions (GE Healthcare, Stockholm, Sweden). Mononuclear cells were diluted in 10 ml of Ex-Vivo10 (fetal bovine serum; Biowhitaker, Europe, Belgium), centrifuged for 10 minutes at 1,400 rpm. Washed mononuclear cells were used fresh for clonogenic assays or gene transfer studies, or cryopreserved under optimized conditions (see below). As an alternative procedure to F-P, BM samples were simply depleted from erythrocytes with HES (Grifols Laboratories, Barcelona, Spain) as previously described.[11] Before lentiviral transduction, erythrocyte-depleted cells were diluted at least 10-fold with Ex-Vivo10.

Cryopreservation of BM cells. Cryopreservation of FA BM cells was conducted under conditions optimized for FA samples.[11] Briefly, F-P or HES separated BM cells were centrifuged and re-suspended in MS1X medium (30% Iscove medium; 50% HES-6%, 20% HSA) with etanercept (10 µg/ml, final concentration). Samples were then maintained at 4 °C. MC2X medium (20% Iscove, 50% HES-6%, 10% dimethyl sulfoxide; 20% HSA) was added slowly to the cell suspension. Cells were aliquoted in cryotubes and cryopreserved at a controlled rate (1 °C/min) in a programmable liquid nitrogen freezer (model CM-2000; Carburos Medica, Barcelona, Spain).

LV-transduction of LCLs and unselected BM from FA patients. For the transduction of mononuclear or erythrocyte-depleted BM cells, samples were re-suspended in Ex-Vivo10 supplemented with 5 ng/ml of thrombopoietin (R&D Systems, Minneapolis, MN), 15 ng/ml of stem cell factor (Peprotech, London, UK), 5 ng/ml of Flt3-L (Invitrogen, Carlsbad, CA), and 5 µg/ml of etanercept (anti-tumor necrosis factor-α fusion protein; Immunex, Seattle, WA) and *N*-acetylcysteine at 100 µmol/l (Sigma-Aldrich, St. Louis, MO). In all experiments, the cells were transduced with LVs previously loaded into retronectin-coated plates. In a first step, non-tissue culture treated plates (Falcon; BD Biosciences, San Jose, CA) were coated with retronectin (20 µg/cm^2; Takara Bio, Shiga, Otsu, Japan) overnight at 4 °C, and then washed once with phosphate buffered saline (PBS), as previously described.[19,38] One milliliter of infective supernatant was then preloaded on retronectin-coated plates (9.6 cm^2 per well) either using a static preloading procedure (either a single 2 hours preloading with 1 ml of supernatant or four preloadings of 30 minutes, using 1 ml in each one), or a dynamic preloading (2 hours of centrifugation of the infective supernatant at 2,500 rpm; see details in **Supplementary Figure S1**). Viral supernatants were then discarded, and LV-preloaded plates were washed with PBS at 37 °C to eliminate residual LV particles. Finally, 3 ml of a cell suspension consisting of 3×10^5 cells/ml were added to plates coated with the LVs, and maintained in the incubator for 16 hours to facilitate the transduction of the samples. After this period, cells were collected, centrifuged at 1,400 rpm for 10 minutes, and the supernatant was discarded. Where described, samples were subjected to additional cycles of transduction. Cells were then cultured in semisolid medium to assess the transduction efficacy on the CFCs. Fourteen days later, the efficacy of transduction of CFCs was determined by evaluating the proportion of colonies positive for EGFP expression by fluorescence microscopy. When samples were transduced with LVs carrying the therapeutic *FANCA* gene, cells were cultured in methylcellulose medium with or without 10 nmol/l mitomycin-C (Sigma Chemicals), in order to determine the proportion of colonies that became resistant to the drug.[19] LCLs were transduced under the same conditions. In this case, however, transduced samples were incubated in liquid cultures for 5 days. The proportion of LCLs expressing the $EGFP^+$ transgene was then determined by flow cytometry (EPICS XL; Coulter Electronics, Hialeah, FL).

In vitro *culture of human BM MSCs.* To generate the MSCs, FA BM cells, either transduced or not, were seeded at a density of 1.5×10^5 cells per cm^2 in flasks (Nalgene Nunc International, Rochester, NY) in MesenCult medium for human cells and MSC-supplements (Stem Cell Technologies, Vancouver, Canada), and incubated at 37 °C in a 5% humidified CO_2 atmosphere, as previously described.[50] The phenotype of cultured MSCs was analyzed by flow cytometry. Cells were harvested by the addition of 0.05% trypsin-EDTA, washed and suspended in PBS with 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO). Aliquots of 5×10^5 cells were incubated for 30 minutes at 4 °C in the dark with conjugated monoclonal antibodies, and washed. Human monoclonal antibodies against CD45, CD29, CD34, CD73, CD90, CD166, CD117, and CD44 (Beckman Coulter, Fullerton, CA) were used for the characterization of the MSCs. Nonspecific fluorescence was determined using aliquots of the cell suspension incubated with isotype monoclonal antibodies.[50] To induce adipogenic differentiation of MSCs, cells were plated at 5×10^4 cells/ml in NH AdipoDiff Medium (Miltenyi Biotech, Bergisch Gladbach, Germany), with medium changes every 3 days. After 21 days, cells were analyzed by Oil Red (Sigma-Aldrich) staining, a lipophilic red dye. For osteogenic differentiation, cells were plated at 3×10^4, cultured in OsteoDiff Medium (Miltenyi Biotech) and every 3 days the medium was changed. After 10 days of culture, the cells were stained for alkaline phosphatase activity with NBT susbstrate (SIGMA FAST BCIP/NBT; Sigma-Aldrich). The analysis of nuclear FANCD2 foci in MSCs was conducted as described in previous studies.[48] Cells were fixed for 15 minutes with 3.7% paraformaldehyde in PBS, followed by permeabilization with 0.5% Triton X-100 in PBS (10 minutes). After blocking in 10% fetal bovine serum, 0.1% NP-40 in PBS (blocking buffer), specific antibodies were added at the appropriate dilution in blocking buffer and incubated for 24 hours at 4 °C. FANCD2 was detected using the affinity-purified rabbit antibody Ab2187 from Abcam, Cambridge, CA. To improve the detection of EGFP-positive cells, an EGFP antibody was also used, Ab291 (from Abcam, Cambridge, UK). Cells were subsequently washed three times in TBS 0.1% NP-40, and species-specific fluorescein or Texas red–conjugated secondary antibodies (Jackson Immunoresearch, Suffolk, UK) were added. After 1 hours at room temperature, three washes were applied, and slides were mounted in Moviol with 4,6-diamidino-2-phenylindole. Images were captured on a Nikon microscope (Nikon, Melville, NY) and processed using a Corel Draw imaging software.

Statistical methods. Statistical analysis was performed using the GraphPad Prism version 4 (GraphPad Software, San Diego, CA). Results

are reported as mean ± SEM. Statistical differences between means were evaluated by using Student's *t*-test. Differences were considered significant at $P \leq 0.05$. Linear regression analysis was applied to determine the relation between proportions of $CD34^+$ cells and CFCs in the BM of FA patients. Similar analyses were conducted to establish correlations between these parameters and different hematological parameters in peripheral blood (**Figure 1**).

SUPPLEMENTARY MATERIAL

Figure S1. Experimental protocols developed for the lentiviral transduction of unselected bone marrow cells from Fanconi anemia patients.

Figure S2. Correlation analysis between the proportion of EGFP-positive colonies and the proportion of mitomycin C resistant colonies after transduction with lentiviral vectors expressing both FANCA and EGFP.

Table S1. Efficacy of Ficoll-Paque and hydroxyethyl starch (HES) to fractionate bone marrow cells from Fanconi anemia patients.

Table S2. Improved transduction of cryopreserved bone marrow progenitors from Fanconi anemia patients after three repetitive cycles of transduction with lentiviral vectors subjected to dynamic preloading.

Table S3. Influence of oxygen concentration on the characteristics of cryopreserved bone marrow progenitors from Fanconi anemia patients transduced with three repetitive cycles as in Table 2.

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