LETTERS
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Successful engraftment of gene-corrected hematopoietic stem cells in non-conditioned patients with Fanconi anemia

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Fanconi anemia (FA) is a DNA repair syndrome generated by mutations in any of the 22 FA genes discovered to date[1,2]. Mutations in *FANCA* account for more than 60% of FA cases worldwide[3,4]. Clinically, FA is associated with congenital abnormalities and cancer predisposition. However, bone marrow failure is the primary pathological feature of FA that becomes evident in 70–80% of patients with FA during the first decade of life[5,6]. In this clinical study (ClinicalTrials.gov, NCT03157804; European Clinical Trials Database, 2011-006100-12), we demonstrate that lentiviral-mediated hematopoietic gene therapy reproducibly confers engraftment and proliferation advantages of gene-corrected hematopoietic stem cells (HSCs) in non-conditioned patients with FA subtype A. Insertion-site analyses revealed the multipotent nature of corrected HSCs and showed that the repopulation advantage of these cells was not due to genotoxic integrations of the therapeutic provirus. Phenotypic correction of blood and bone marrow cells was shown by the acquired resistance of hematopoietic progenitors and T lymphocytes to DNA cross-linking agents. Additionally, an arrest of bone marrow failure progression was observed in patients with the highest levels of gene marking. The progressive engraftment of corrected HSCs in non-conditioned patients with FA supports that gene therapy should constitute an innovative low-toxicity therapeutic option for this life-threatening disorder.

Although the very low number of hematopoietic stem cells (HSCs) in patients with Fanconi anemia (FA)[7] has limited the collection of HSCs in previous gene therapy trials[8–10], the proliferative advantage observed in naturally reverted hematopoietic stem and progenitor cells (HSPCs) from mosaic patients with FA[11–14] suggested that the infusion of low numbers of gene-corrected HSCs might be sufficient for their engraftment after autologous transplantation. Nevertheless, none of the gene therapy trials conducted to date have shown engraftment of corrected HSCs in patients with FA[9,10]. Several factors may account for these negative results, including the collection of low numbers of HSCs from bone marrow (BM) or granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood (PB), the use of prolonged transduction protocols with gamma-retroviral vectors, or the absence of patients' conditioning before the infusion of transduced cells (see review in ref. [15]).

The use of CD34$^+$ cells mobilized with G-CSF and plerixafor, together with the short transduction of these cells with *FANCA* lentiviral vectors, enabled us to demonstrate the repopulating potential of gene-corrected FA subtype A (FA-A) HSCs in a xenogenic transplantation model[16]. On the basis of our preclinical observations, we developed the current clinical trial in which gene-corrected HSCs were reinfused in patients with FA without any cytotoxic conditioning regimen.

CD34$^+$ cells were obtained from pediatric patients (aged 3–6 years) before the development of severe BM failure (BMF) (see Extended Data Fig. 1 and the main inclusion criteria of the FANCOSTEM-1 HSC mobilization trial in the Methods). In contrast, because the primary objective of the FANCOLEN-1 gene therapy trial was to evaluate the safety of the therapeutic strategy, only

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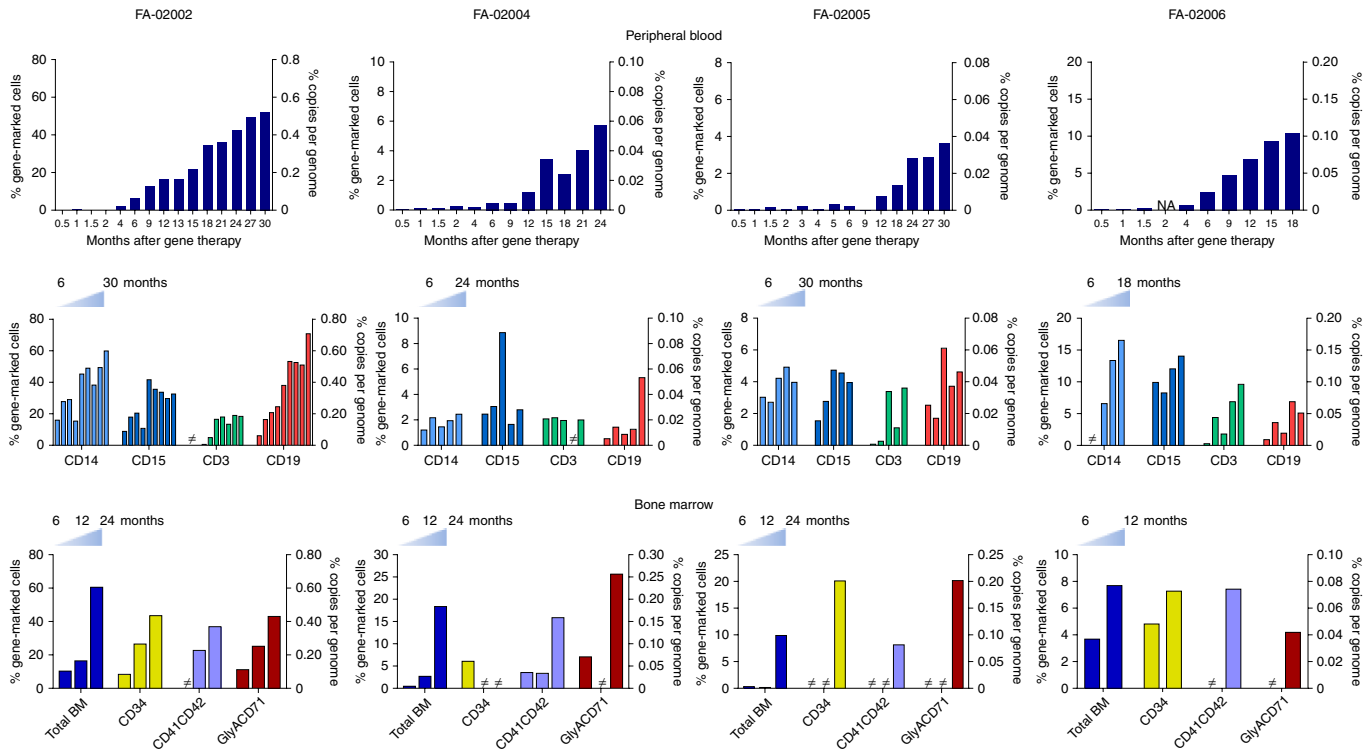

Fig. 1 | Progressive engraftment of gene-corrected cells in non-conditioned patients with FA-A treated by gene therapy. Kinetics of engraftment of corrected leukocytes (top) and purified PB populations (middle), represented either as a percentage of corrected cells (left axes) or VCNs cell^{-1} (right axes) at different times post-infusion. Gene-corrected cells in purified PB cells were analyzed every 3 months from month 6 post-infusion. Bottom: proportion of corrected cells in BM subpopulations at 6, 12 and 24 months after infusion of corrected cells. Percentages of gene correction in PB and BM were calculated based on the fact that VCNs in transduced CD34$^+$ cells were always lower than 1.0 copy cell^{-1} at 14 d post-transduction (see Extended Data Fig. 2). VCNs were determined in two to three independent runs per sample, depending on the amount of extracted DNA. In purified PB and BM subpopulations with limited amounts of DNA, single VCN determinations could be made in some instances, always in parallel with control DNA samples harboring known copies of the provirus. NA, not analyzed. Hashtags represent samples not evaluable since the quantity of DNA was under the detection limit for qPCR amplification of the human albumin gene.

patients with evidence of BMF were infused with autologous gene-corrected HSPCs (see Extended Data Fig. 1 and inclusion criteria of FANCOLEN-1 in the Methods). In this report, data corresponding to the 18- to 30-month follow-up of the initial four patients infused between January 2016 and March 2017 are shown. In none of these patients have serious adverse events associated with the investigational therapy been observed.

In two patients (FA-02002 and FA-02004), CD34$^+$ cells were collected and cryopreserved for 22 and 20 months, respectively, until patients met the inclusion criteria of the gene therapy protocol (Extended Data Fig. 1). Cryopreserved CD34$^+$ cells were then thawed, transduced and infused. In patients FA-02005 and FA-02006, PB counts met the criteria required for both CD34$^+$ cell collection and the gene therapy protocols (Extended Data Fig. 1). In these patients, freshly collected CD34$^+$ cells were transduced and infused without cryopreservation. To evaluate transduction efficacies in infused products, the mean vector copy number (VCN) per cell (VCN cell^{-1}) was calculated, and analyses of the proportion of mitomycin C (MMC)-resistant colony-forming cells (CFCs resistant to 10 nM MMC) were carried out in aliquots of transduced samples (Extended Data Fig. 2). Based on VCNs and analyses of HSPCs conducted after transduction, numbers of corrected HSPCs infused per patient were determined (Extended Data Fig. 2).

Patient FA-02002 was infused with an estimated number of 250,000 corrected CD34$^+$ (cCD34$^+$) cells kg^{-1} and 14,000 corrected CFCs (cCFCs) kg^{-1}. Although a very low proportion of marked cells was observed in PB during the first 2 months post-infusion (<0.3%), progressive increases in gene marking were observed thereafter, reaching levels of 55% in PB leukocytes and 70% in B lymphocytes at the most recent follow-up (30 months; Fig. 1). In BM, progressive increases in gene marking were observed in all lineages, reaching values of 37–60% at 24 months post-infusion. Importantly, at this time point, 43.5% of BM CD34$^+$ cells were gene marked, suggesting the engraftment of very primitive corrected HSCs.

Patient FA-02004 was infused with a lower number of transduced CD34$^+$ cells (160,000 cCD34$^+$ cells kg^{-1} and 7,300 cCFCs kg^{-1}). In this patient, the detection of corrected cells in PB and BM was markedly delayed compared with patient FA-02002. However, progressive increases in the proportion of corrected cells were also observed, reaching levels of 2–9% in PB and 15–25% in BM at 24 months post-infusion (Fig. 1). In patient FA-02005, 220,000 cCD34$^+$ cells kg^{-1} and 2,800 cCFCs kg^{-1} were infused. The kinetics of engraftment in this patient was similar to that observed in patient FA-02004, reaching values of 4–6 and 8–20% in PB and BM cell lineages, respectively, at the most recent evaluation (30 and 24 months post-infusion, respectively). More recently, patient FA-02006 was infused with 410,000 cCD34$^+$ cells kg^{-1} and 160,000 cCFCs kg^{-1}. The higher number of progenitor cells infused in this patient is believed to be associated with the modified immunoselection process that was used in one of the apheresis products to improve the yield of purified CD34$^+$ cells (see Methods). The kinetics of engraftment of corrected cells ranged between that observed in patient FA-02002 and that in patients FA-02004 and FA-02005, with gene-marking levels of 5–17% in PB and 4–8% in BM at the most recent evaluation (18 and 12 months post-infusion, respectively; Fig. 1).

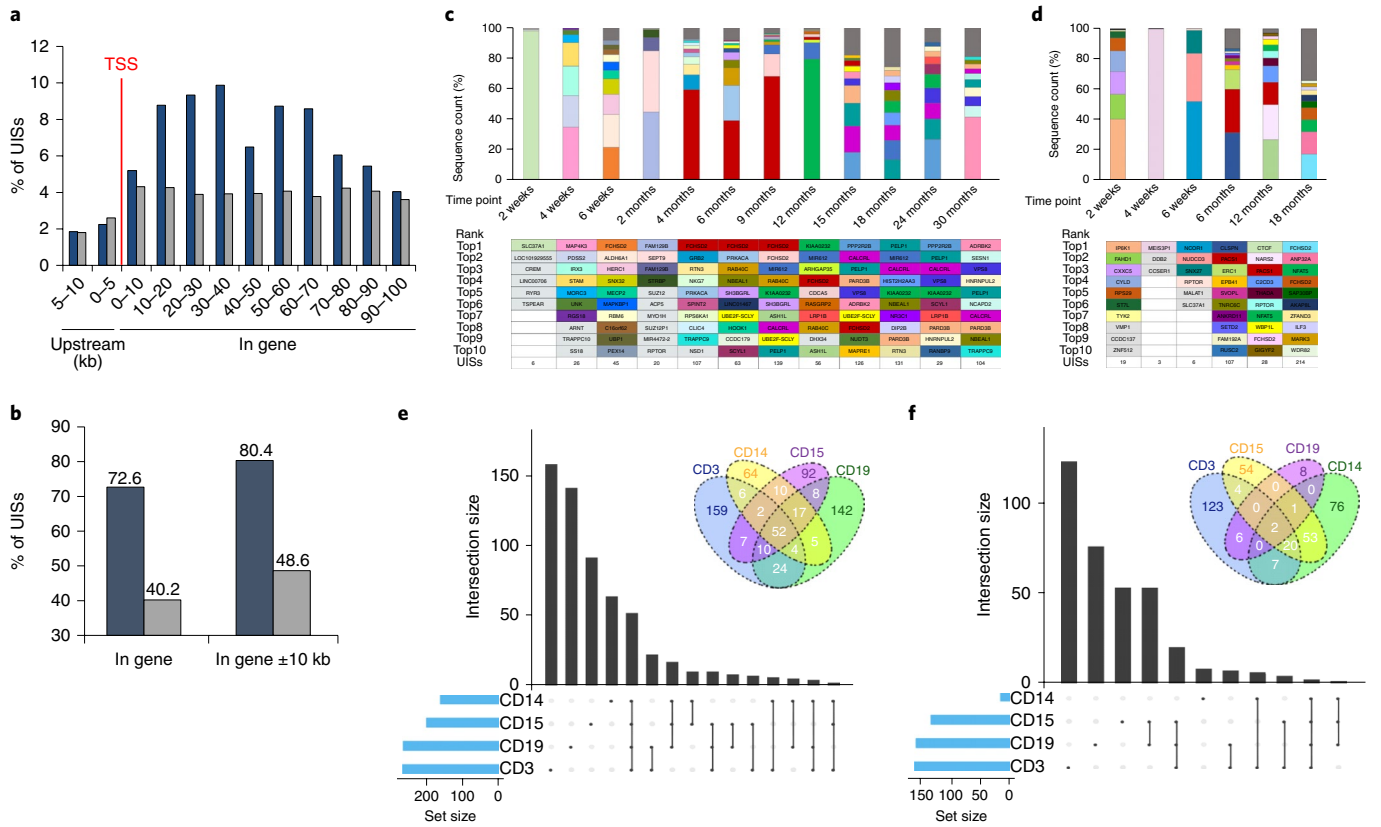

Fig. 2 | FANCA lentiviral vector integration profile and genome-wide mapping of UISs after hematopoietic reconstitution of patients with FA treated by gene therapy. a, Distribution of the 2,001 UISs determined in PB samples from the four patients with FA treated by gene therapy in genes and in genes ± 10 kb. TSS, transcription start site. **b**, A total of 1,453 UISs were observed in gene regions (72.6%), while 1,608 UISs (80.4%) were observed in gene ± 10 kb regions. No clustering around TSS regions was observed. Bars in gray represent percentages corresponding to a random distribution of UISs, while blue bars represent actual proportions of UISs determined in the four patients with FA included in the trial. **c**,**d**, Top: the ten most represented UISs in PBMCs from patients FA-02002 (**c**) and FA-02006 (**d**) at different times after gene therapy. UISs detected at more than one time point, or with a contribution higher than 1% at one time point, are marked with colors. UISs marked in dark gray are those not represented in the top ten rank. Bottom: the RefSeq genes nearest to the UISs are listed in the tables. The top ten clones marked in light gray indicate clones with a contribution lower than 1%. The total numbers of UISs are indicated at the bottom of each column. **e**,**f**, UpSet plots (left) and Venn diagrams (right) showing the absolute numbers of UISs shared by myeloid (CD14 and CD15) and T- and B-lymphoid lineages (CD3 and CD19) from patients FA-02002 (**e**) and FA-02006 (**f**). These analyses include UISs obtained over the different observation time points. The blue bars in the UpSet plots represent the size of each set. The description of each set is listed on the right side of each bar. The intersections between the sets reflect the numbers of UISs shared between different lineages.

None of the tested BM samples from gene therapy-treated patients showed cytogenetic abnormalities, as deduced from G-banding and fluorescence in situ hybridization (FISH) analyses of chromosomes 1q, 3q and 7q and comparative genomic hybridization (array CGH) analyses (Extended Data Figs. 3 and 4; array CGH files are available at ArrayExpress (European Bioinformatics Institute) with the reference E-MTAB-8139; https://www.ebi.ac.uk/arrayexpress/). Additionally, no mutations were found in a myeloid malignancy 85-gene panel that includes *RUNX1* (not shown). These studies strongly suggest that genetic abnormalities potentially indicative of a malignant condition[17,18] were not present in any of the treated patients. Since this trial was conducted in non-conditioned patients with FA-A, T cell-mediated immune responses against FANCA were investigated using an interferon-γ (IFN-γ) enzyme-linked immune absorbent spot (ELISpot). Importantly, no immune reactivity against FANCA was detected in any sample analyzed up to 13 months post-infusion (not shown).

To evaluate the clonal nature of engrafted HSCs and potential genotoxic effects of the therapeutic provirus, insertion-site analysis (ISA) was conducted in PB cells at multiple time points post-infusion. On average, 72.6% of the unique integration sites (UISs) occurred within genes (80.4% in genes ± 10 kb), and no preferential integrations were found in close proximity to transcription start sites (see Fig. 2a,b), consistent with previous findings observed in other lentiviral-vector-mediated gene therapy trials[19–23], as well as in our preclinical studies with the phosphoglycerate kinase-FANCA-mutated woodchuck hepatitis virus post-transcriptional regulatory element (PGK-FANCA-WPRE*) lentiviral vector[24]. Longitudinal ISA for patients with the highest levels of gene marking (patients FA-02002 and FA-02006) showed an oligoclonal/polyclonal reconstitution pattern with succession of marked clones over time (Fig. 2c,d). Levels of polyclonal reconstitution in these two patients (gray bars) were more substantial subsequent to the first year post-infusion, indicating that progressive increases in the proportion of corrected PB cells were not associated with the dominance of one or a small number of clones with genotoxic insertions, but rather with the progressive expansion of a large number of corrected repopulating clones. ISA conducted in these patients at earlier time points post-infusion, and in patients FA-02004 and FA-02005 (characterized by low levels of gene marking), identified a substantially reduced number of UISs, which markedly fluctuated over the follow-up (Extended Data Fig. 5). Importantly, integrations in genes such as *LMO2*, *CCND2* and *MECOM*, which were previously associated with the occurrence of serious side events, were not detected.

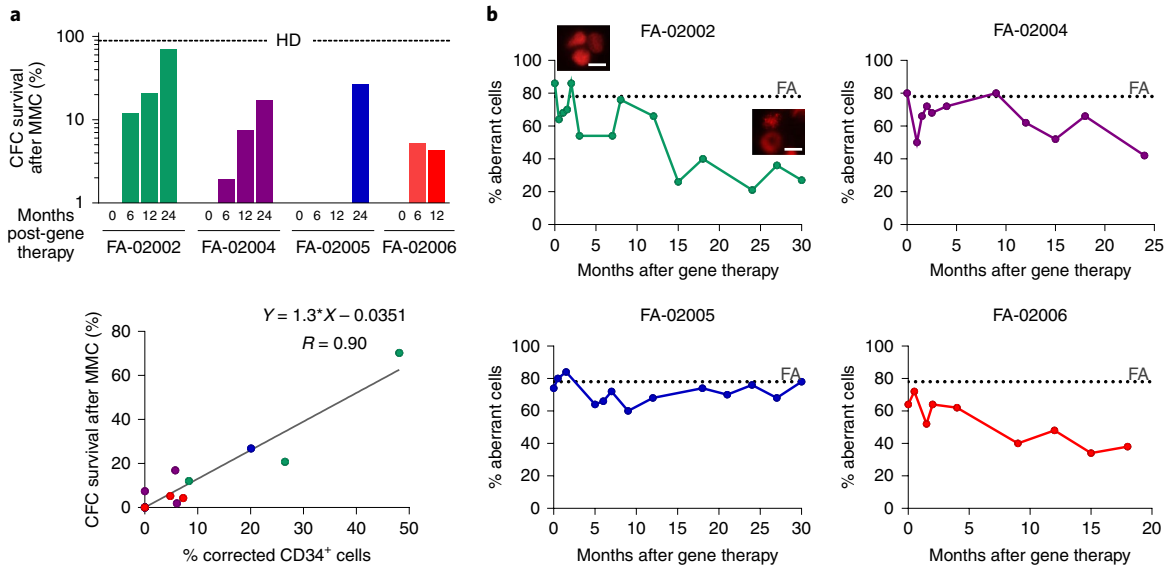

Fig. 3 | Phenotypic correction of BM progenitors and PB T cells in patients with FA treated by gene therapy. a, Top: survival of BM CFCs after MMC (10 nM) exposure before and after infusion of transduced CD34+ cells in non-conditioned patients with FA. The mean value corresponding to healthy donors (HD) is also shown (dashed line). Bottom: correlation between the survival of CFCs after MMC administration and percentages of corrected CD34+ cells determined in the same BM samples. A linear regression analysis was conducted. Each color represents one individual patient (same colors as those used in **a**). CFC values represent mean ± s.e.m. values corresponding to the scoring of three plates seeded with BM cells before and after infusion of transduced CD34+ cells. **b**, Proportion of PB T cells with chromosomal breaks after in vitro exposure to DEB (0.1 μg ml^{-1}) at different times after gene therapy. For comparison, the historical mean value corresponding to patients with FA is shown (dashed lines). Between 25 and 50 metaphases were analyzed at each time point. Representative pictures of FANCD2 immunostainings of MMC-treated PB T cells from patient FA-02002 before and 30 months after gene therapy are shown. In total, 200 cells were analyzed. Scale bars: 5 μm.

Due to the high proportion of corrected cells observed in patient FA-02002, additional ISA was carried out with sorted PB cells. The UpSet plot[25] and Venn diagram shown in Fig. 2e revealed that many of the UISs identified in this patient were detected in one cell lineage (mainly in T and B cells). Nevertheless, several other UISs were identified in two, three and even four purified cell lineages, showing engraftment of gene-corrected multipotent HSCs. Similar results were obtained in patient FA-02006 (Fig. 2f), although in this case a lower proportion of multipotent repopulating clones were identified, probably due to the lower level of gene marking and shorter follow-up (18 months post-infusion).

To investigate whether increases in the proportion of corrected hematopoietic cells were associated with the reversion of their characteristic FA phenotype, CFC survival of BM progenitor cells after MMC exposure, and the proportion of PB T cells with chromosomal breaks after exposure to diepoxybutane (DEB), were assessed. In patient FA-02002, survival after MMC administration increased up to 70% at 24 months post-infusion, which was very close to values identified in healthy donors (Fig. 3a). Additionally, a substantial increase in the absolute number of BM CFCs was observed at the most recent follow-up visit (24 months post-infusion), suggesting expansion of the HSPC compartment (Extended Data Fig. 6). In the three other patients, progressive increases in the MMC resistance of CFCs were also observed. Importantly, a strong correlation between the survival of CFCs after MMC administration and the proportion of corrected BM CD34+ cells was observed (Fig. 3a), indicating that levels of gene correction accounted for the phenotypic correction of BM HSPCs.

Analyses of chromosomal breaks in PB T cells exposed to DEB showed a progressive decrease in the proportion of cells with aberrant chromosomes in patient FA-02002 (Fig. 3b). Consistent with this result, we observed the presence of FANCD2 foci in T cells 30 months after gene therapy—a phenomenon that is strictly dependent on a functional FA pathway (Fig. 3b). Additionally, progressive decreases in the proportion of T cells with aberrant chromosomes were noted in patients FA-02004 and FA-02006, although these were not as pronounced as those observed in patient FA-02002.

Hematological analyses performed in patients with FA before the infusion of gene-corrected cells indicated progressive decreases in at least two PB lineages (Fig. 4a). However, stabilized neutrophil counts and hemoglobin levels were observed in patient FA-02002 after month 6 post-infusion, and a similar trend was noted in patients FA-02005 and FA-02006. Prophylactic platelet transfusions were only required in patient FA-02005 at days 32 and 36, and at months 12 and 18 post-gene therapy, while in patients FA-02002, FA-02004 and FA-02006, stable but relatively low platelet counts were observed.

Finally, based on the analyses of the proportion of corrected leukocytes (Fig. 1), and considering the numbers of PB leukocytes, the total number of corrected and uncorrected leukocytes could be determined in gene therapy-treated patients at different times post-infusion (Fig. 4b). In contrast with the continuous decline of uncorrected leukocytes, a progressive increase in the total number of corrected leukocytes was observed soon after the infusion of transduced cells in all treated patients, especially in patients with the highest proportion of corrected cells (FA-02002 and FA-02006).

Discussion

Previous studies have shown the efficacy of hematopoietic gene therapy in FA mouse models[24,26–31]. Nevertheless, none of the clinical trials conducted to date have indicated sustained engraftment of autologous gene-corrected HSCs in patients with FA[9,10]. The data presented in this clinical study show progressive engraftment of gene-corrected autologous HSCs in patients with FA, despite the absence of conditioning in any of these patients. The most robust results were noted in patients FA-02002 and FA-02006, who were infused with the highest numbers of transduced HSPCs. Additionally, the high number of primitive hematopoietic precursors (CD34+CD38− cells) observed in the BM of patient FA-02002 before mobilization (Extended Data Fig. 1) suggests the relevance of HSC quality for the engraftment of corrected cells.

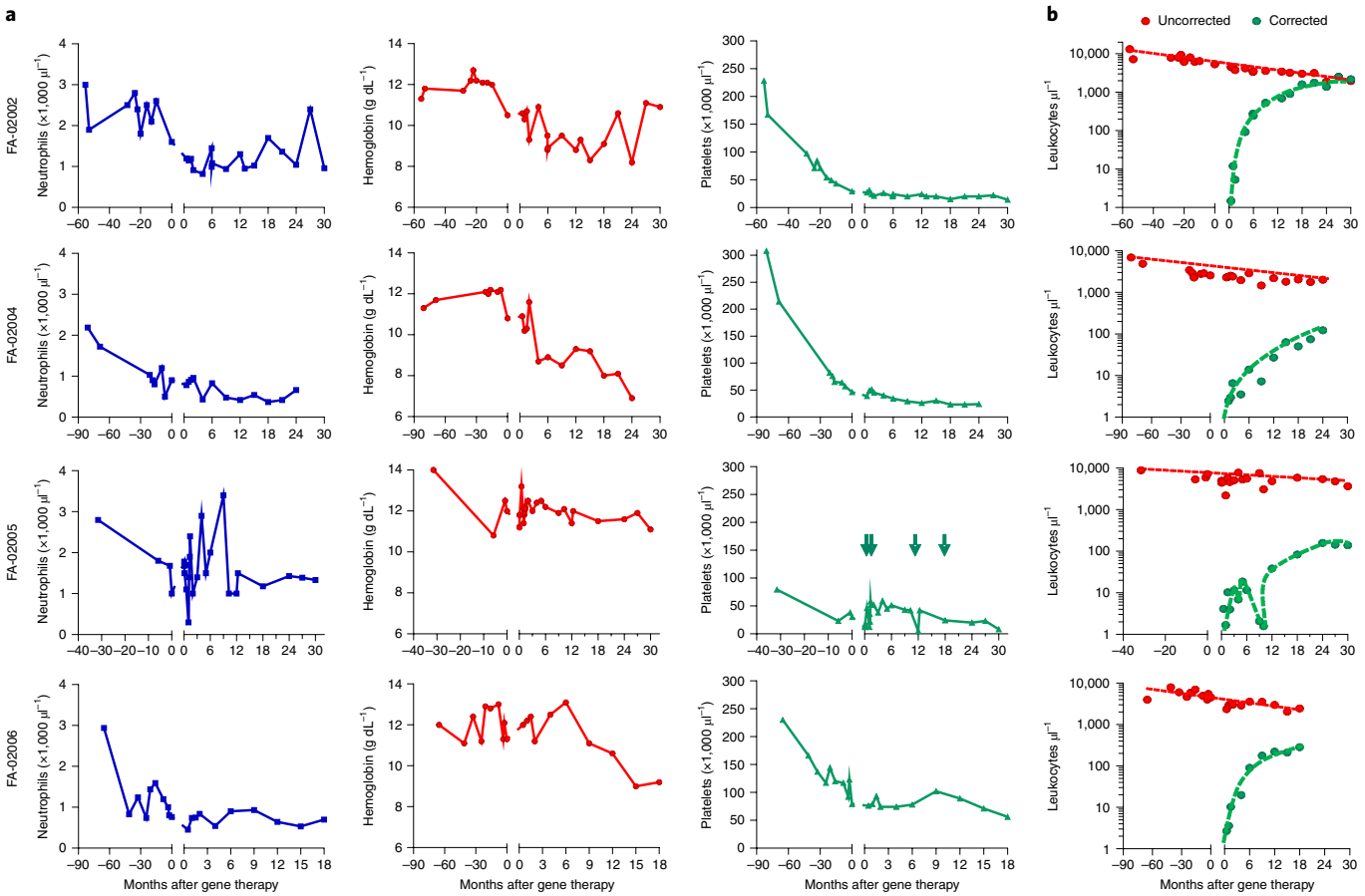

Fig. 4 | PB cell counts in patients with FA before and after gene therapy. a, Evolution of neutrophils (left; blue), hemoglobin levels (middle; red) and platelets (right; green) in PB of patients with FA before and after gene therapy. Green arrows indicate the administration of prophylactic platelet transfusions. **b**, Kinetics of uncorrected (red) and corrected leukocytes (green) in the PB of patients with FA before and after gene therapy.

Detailed cytogenetic studies and array analyses of myeloid cancer genes have strongly suggested the absence of leukemogenesis events in any of the gene therapy-treated patients. Importantly, ISA showed that the progressive engraftment of gene-corrected repopulating cells was not associated with genotoxic insertions in FA HSCs, but rather with the normalized phenotype of these cells. Compared with the very high number of HSC clones observed in gene therapy trials of other monogenic diseases[19–23], our ISA demonstrated a more limited clonality, consistent with the low number of corrected CD34$^+$ cells that could be infused in these patients with FA, and also with the absence of genotoxic conditioning in this FA gene therapy trial. Future improvements enabling the infusion of higher numbers of gene-corrected HSCs are likely to augment the clonal diversity and also accelerate the engraftment of corrected hematopoietic cells in patients with FA.

In addition to the progressive phenotypic correction of the hematopoietic cells, analyses of PB cell counts in our patients with FA suggest a halt in the progression of BMF (Fig. 4a)—an observation that was particularly apparent in patient FA-02002. Although no evident increases in PB cell counts have been observed in our patients to date, the sustained increase in the total number of corrected leukocytes (Fig. 4b), together with the identification of corrected CD34$^+$ cells and multipotent HSCs in treated patients (see Figs. 1 and 2), suggest the hematological recovery of these patients over the long term. This hypothesis is based on previous studies[11–14], as well as our own unpublished observations, showing that the hematological recovery of mosaic patients with FA also constitutes a slow process that requires years of evolution. As may also occur in mosaic patients, the incomplete replacement of mutated FA hematopoietic cells might result in the accumulation of mutations in uncorrected cells, potentially leading to myelodysplastic syndromes or leukemia. Nevertheless, no genetic or cytogenetic abnormalities have been observed in any of our gene therapy-treated patients through 30 months of follow-up. Additionally, preliminary observations indicate that the leukemia incidence in mosaic patients with reversions in HSCs is reduced compared with non-mosaic patients (J. Surrallés and J. D. Schwartz, personal communication).

Potential advantages of the current gene therapy approach in FA are several-fold. First, we demonstrate the clinical feasibility of correcting the genetic defect in a very challenging disease that causes BMF and leukemia, and which constitutes a highly unmet clinical need. Since gene therapy in FA appears feasible and safe in the absence of cytotoxic conditioning, it could be implemented early in the disease course to avert BMF and other hematological complications through a therapeutic intervention that only requires brief hospitalization. Second, in contrast with allogeneic transplantation, the proposed autologous gene therapy is not likely to increase the subsequent incidence of solid tumors, since neither genotoxic conditioning nor graft-versus-host disease—the main parameters associated with the increased incidence of solid tumors in transplanted patients with FA[32,33]—will play any role in treated patients. Finally, in case solid tumors appear, an improved hematological response to chemotherapy and a healthier immune system would be expected in gene therapy-treated patients, thus allowing the administration of more efficient anticancer therapies.

Taken together, our results strongly suggest that gene therapy will offer a low-toxicity option that may prove particularly impactful for

patients with FA. Based on our observations demonstrating engraftment of corrected HSCs in non-conditioned patients, further improvements will focus on the infusion of higher numbers of corrected HSCs, and potentially the use of non-genotoxic conditioning regimens.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41591-019-0550-z.

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Author contributions

P.R., S.N., W.W., R.S.-D., R.M.P., J.C.S., M.B., E.M., N.W., R.S., M.L.Lamana, R.M.Y., J.A.C., Y.G., F.J.R.-R., L.A., O.A., A.R., G.G., M.L.Lozano., L.C., M.H., E.G., I.G., J.B., C.D.H., A.G., J.Surrallés, J.Soulier and M.S. performed the experimental studies and provided critical materials and reagents. R.H., N.G.A., R.L. and A.C. diagnosed patients and included them in the clinical trial. C.D.H. and J.Sevilla conducted the clinical trial. P.R., S.N., J.Sevilla and J.A.B. wrote the manuscript. J.D.S. reviewed the manuscript. P.R., S.N., C.D.H., J.Sevilla and J.A.B. designed the study. All authors discussed the results and contributed to the preparation of the manuscript.

Competing interests

The Hematopoietic Innovative Therapies division at CIEMAT receives funding and has licensed the PGK-FANCA-WPRE* lentiviral vector to Rocket Pharmaceuticals. J.A.B., J.Sevilla and J.C.S. are consultants for Rocket Pharmaceuticals. J.D.S. is Medical Director of Rocket Pharmaceuticals. P.R., S.N., J.A.C., J.C.S., J.Sevilla, G.G. and J.A.B. are inventors on patents on lentiviral vectors filed by CIEMAT, CIBERER and Fundación Jiménez Díaz, and may be entitled to receive financial benefits from the licensing of such patents. M.S. is co-founder and CEO of GeneWerk. W.W. is an employee of GeneWerk. The remaining authors declare no competing interests.

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Methods

Patients and CD34⁺ cell processing. Mobilized PB HSCs were collected from four pediatric patients with FA-A (aged 3–6 years) before the development of severe BMF, as described in the FANCOSTEM clinical trial protocol (ClinicalTrials.gov, NCT02931071; European Clinical Trials Database, 2011-006197-88), complying with all relevant ethical regulations and approved by the ethics committees at Hospital Vall d'Hebron in Barcelona and Hospital del Niño Jesús in Madrid. For inclusion, patients had to have at least one hematological parameter above the following thresholds: hemoglobin > 8 g dl^{-1}; neutrophils > 750 cells µl^{-1}; or platelets > 30,000 cells µl^{-1}. None of the patients had been transfused for 3 months before being treated with mobilizing drugs. None of them had ever received androgen treatment. Patients were mobilized by the administration of G-CSF (Neupogen, Amgen; 12 µg kg^{-1} every 12 h for 6–7 d) and plerixafor (Mozobil; Genzyme; 240 µg kg^{-1} d^{-1} for 2–3 d). Collected cells were immediately processed for CD34⁺ cell immunoselection. This procedure was performed as described by the Joint Accreditation Committee of the International Society for Cellular Therapy (Europe) and the European Society for Blood and Marrow Transplantation in an accredited unit. Cells were processed with the automated CliniMACS device (Miltenyi Biotec), according to the manufacturer's standard protocol with minor changes. The collected product was washed using the washing buffer described by the manufacturer (namely, CliniMACS phosphate buffered saline (PBS)/EDTA buffer), supplemented with human albumin (20% solution from Grifols)) at a final concentration of 5%. Manual processing was performed using refrigerated centrifuges according to procedures described in the CliniMACS manual. Cells were incubated in all cases with one vial of CliniMACS CD34 Reagent (composed of iron-dextran superparamagnetic particles conjugated to a monoclonal anti-CD34 antibody) for 30 min with slow agitation. Before incubation with the monoclonal antibody, and to avoid non-specific binding, 3 ml human immunoglobulin G (Flebogamma; Grifols) was added to the cell suspension bag. After incubation, cells were washed again and finally diluted to a standard volume of 150 ml. The bag with the washed and labeled cells was hung on the device for automated selection of CD34⁺ cells using software version 2.4 and selection program 1.2. In one of the two apheresis procedures obtained from patient FA-02006, the selection program was modified, using program 3.1 (designed for T cell depletion). According to this modification, the tubing set was also changed to the one designed for T cell depletion (DTS; 261–01) to collect the target cells (CD34⁺ cells) into the negative fraction bag. For patients FA-02002 and FA-02004, purified CD34⁺ cells were cryopreserved following standard operating procedures for hematopoietic progenitor cell cryopreservation with 10% dimethyl sulfoxide and autologous or allogeneic plasma. Immunophenotypic analyses of HSPCs were conducted by flow cytometry using anti-CD45 APC, CD34 PE and CD38 FITC antibodies (all from Becton Dickinson). Fluorochrome-matched isotypes were used as controls. Cells positive for 4',6-diamidino-2-phenylindole (DAPI; Roche) were excluded from the analysis. Analysis was performed using FlowJo software.

Before the infusion of gene-corrected cells, patients' parents provided written informed consent corresponding to the gene therapy trial (FANCOLEN-1; ClinicalTrials.gov, NCT03157804; European Clinical Trials Database, 2011-006100-12). This also complied with all of the relevant ethical regulations, and was approved by the ethics committees at Hospital Vall d'Hebron in Barcelona and Hospital del Niño Jesús in Madrid. Initially, five patients were approved by the Regulatory Agency. When the first four patients reported in this study were treated, a new cohort of six patients was approved. For inclusion, patients had to have at least one of the following parameters: hemoglobin < 8 g dl^{-1}; neutrophils < 750 cells µl^{-1}; or platelets < 30,000 cells µl^{-1}. After treatment of patient FA-02002, an amendment in the inclusion criteria was approved, and thresholds for neutrophil and platelet cell numbers were modified to <1,000 cells µl^{-1} and <50,000 cells µl^{-1}, respectively. The main characteristics of the patients at the points of collection and infusion of corrected cells are shown in Extended Data Fig. 1.

Lentiviral transduction of immunoselected CD34⁺ cells. Due to the fragility of FA CD34⁺ cells, a modified transduction procedure was carried out at the Clinistem Facility (Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid). Enriched CD34⁺ cells were pre-stimulated for 8–10 h at 1 × 10^6 cells ml^{-1} in X-VIVO 20 medium (Stemcell Technologies) supplemented with 100 ng ml^{-1} SCF, TPO, Flt3 and 20 ng ml^{-1} IL-3 (all from Peprotech), 10 µg ml^{-1} anti-TNFα (Enbrel (etanercept)) and 1 mM *N*-acetylcysteine (Pharmazam), under hypoxic conditions (5% O_2) using VueLife FEP cell culture bags (CellGenix) pre-coated with 5 µg cm^{-2} RetroNectin (Takara). After pre-stimulation, cells were transduced with 3 × 10^8 IU ml^{-1} of the lentiviral vector produced under good manufacturing practices (GMP PGK-FANCA.WPRE* lentiviral vector)[34] (orphan drug designations EU/10/822 and USA/#16–5193), manufactured at Genethon and YposKesi for 12–14 h in the presence of 8 µg ml^{-1} protamine sulfate at a concentration of 5 × 10^5 cells ml^{-1}.

Autologous infusion of transduced FA-A CD34⁺ cells. Between 2 and 3 h after transduction, cells were infused over a 30-min infusion period. No medication was administered before infusion except for the first patient (FA-02005) who received intravenous paracetamol for fever prophylaxis. Since no adverse reactions related to the infusion were recorded for this patient, we did not administer any prophylactic treatment for the subsequent patients. Cells were infused through a central venous catheter in those patients treated with fresh products, and through a peripheral venous line in those who received thawed cells. Vital signs, such as heart rate, respiratory rate and blood pressure, were recorded every 5 min, and temperature was recorded every 15 min, until 30 min after the infusion. Patients were monitored for any adverse events in the hospital for 72 h after infusion. During this period, vital signs were recorded at least three times per day, and vital organ functions were monitored every day.

CFC assays. To assess the number of CFCs, transduced CD34⁺ cells or BM samples from treated patients were cultured for 14 d at 37 °C under 5% CO_2 and 5% O_2 in methylcellulose medium (#H4434; MethoCult) supplemented with 10 µg ml^{-1} anti-TNFα and 1 mM *N*-acetylcysteine. The proportion of MMC-resistant CFCs was calculated based on colony numbers scored in the absence and presence of 10 nM MMC (Sigma–Aldrich).

ELISpot for IFN-γ release. The immune reactivity to human FANCA (hFANCA) protein was evaluated in patients' peripheral blood mononuclear cells (PBMCs) using an IFN-γ ELISpot assay (Mabtech AB). Briefly, 96-well plates coated with the primary anti-IFN-γ monoclonal antibody (1-D1K clone) were washed four times with PBS and blocked with 100 µl complete RPMI medium containing 10% human AB serum, 2 mM glutamine, 50 UI ml^{-1} penicillin and 50 µg ml^{-1} streptomycin, for 30 min at room temperature. Then, 1.6 × 10^4, 1 × 10^5 or 2 × 10^5 PBMCs in 100 µl of complete medium were added per well and incubated with either the medium alone, 2 µg ml^{-1} HBsAg adw (ProSpec), 40 ng ml^{-1} phorbol myristate acetate + 1 µM ionomycin or 2 µg ml^{-1} recombinant hFANCA (Sino Biological) in duplicates. Cells were incubated for 48 h at 37 °C in 5% CO_2. Wells were washed five times with PBS, followed by a 2-h incubation with 100 µl of the secondary antibody (biotinylated anti-IFN-γ monoclonal antibody; clone 7-B6-1) diluted at 1:200 in PBS + 0.5% FCS. Plates were washed five times with PBS, and avidin-bound alkaline phosphatase was added to the wells and incubated for 1 h at room temperature. The plates were washed five times, followed by 5-min incubation with the alkaline phosphatase substrate BCIP/NBT-plus to develop the reaction. Tap water was added to stop the reaction. The spots were counted using an ELISpot reader (AID Autoimmun Diagnostika). An anti-FANCA immune response was considered positive if there was at least a difference of six spots or more after subtracting the mean spot number of the negative controls (medium alone) from that of the hFANCA-stimulated cells.

Chromosomal instability test in PB T cells. Analysis of the percentage of aberrant cells after DNA damage with DEB was carried out as previously described[35]. Briefly, PB cells were cultured for 24 h in RPMI supplemented with 15% fetal bovine serum, 1% antibiotics, 1% L-glutamine and 1% phytohemagglutinin (all reagents from Gibco). After 24 h, a portion of the cells was treated with DEB at a final concentration of 0.1 µg ml^{-1} (Sigma–Aldrich). Then, 46 h after DEB treatment, colcemid (0.1 µg ml^{-1}) was added. Metaphase spreads were obtained 2 h later and stained with Giemsa. Analysis of 25–50 metaphases of DEB exposed or non-exposed cultures were conducted in a Zeiss Imager M1 microscope coupled to a computer-assisted metaphase finder (MetaSystems). The criteria for the determination and quantification of chromosome breakage were previously described[35].

Immunofluorescence of nuclear FANCD2 foci. PB samples from patient FA-02002, depleted from erythrocytes with hydroxy-ethyl starch (HES) (Grifols Laboratories), were stimulated on non-treated plates coated with 30 µg ml^{-1} purified anti-hCD3 and anti-hCD28 (Pharmingen) monoclonal antibodies. Cells were cultured in Iscove's modified Dulbecco's medium with 20% fetal bovine serum (Gibco) with antibiotics for 4 or 5 d to obtain proliferating T cells. PB samples were seeded in chamber slides (Nunc; Sigma–Aldrich) previously coated with 5 µg cm^{-2} RetroNectin (Takara). Then, 24 h later, cells were incubated in the absence or presence of 40 nM MMC (Sigma–Aldrich). Cells were washed with PBS and fixed with 3.7% paraformaldehyde (Sigma–Aldrich) for 15 min at room temperature. After permeabilization, cells were incubated overnight with the antibody against human FANCD2 (ab2187-50; Abcam) and then incubated for 1 h with the secondary antibody immunoglobulin G against rabbit, conjugated with Alexa Fluor 594 (ab150080; Abcam; 1:1,000 dilution) and DAPI (Roche). Samples were visualized with an Axioplan 2 imaging fluorescence microscope (Carl Zeiss) with a 0.17 mm objective and 100×/1.45 NA magnification. Images were captured with an AxioCam MRm (Carl Zeiss), and processed with AxioVision 4.6.3 (Carl Zeiss) and Corel Photo-Paint 11 (Corel).

Cytogenetic and FISH analyses. BM samples were cultured and harvested for cytogenetic analysis according to established methods. Chromosome slides were G-banded. Chromosomal aberrations are described according to the guidelines of an International System for Human Cytogenetic Nomenclature (ISCN 1995). At least 10 (usually 20) BM cells were analyzed. FISH analyses were conducted using the following FISH probes: XL CDKN2C/CKS1B (1p32/1q21) (MetaSystems), Vysis RPN1/MECOM DF FISH Probe Kit (Abbott Molecular) and Vysis D7S486/CEP 7 FISH Probe Kit (Abbott Molecular). In total, 200 interphase nuclei per FISH were scored.

Targeted next-generation sequencing. Targeted gene-sequencing libraries were prepared with genomic DNA obtained from BM samples using SureSelectXT Target Enrichment (Agilent Technologies). We used a custom 'myeloid neoplasm' gene panel that included *ASXL1, ASXL2*, *ATM*, *ATR*, *BCOR*, *BCORL1*, *BRAF*, *BRCA2, BRCC3*, *CBL*, *CEBPA*, *CHEK2*, *CREBBP*, *CSF3R*, *CTCF*, *CUX1*, *DDX41*, *DNMT3A*, *EP300*, *ERCC6L2*, *ETNK1*, *ETV6*, *EZH2*, *FLT3*, *GATA2*, *HRAS, IDH1*, 1*DH2*, *IRF1*, *JAK2*, *KDM6A*, *KIT*, *KMT2A/MLL*, *KMT2D/MLL2*, *KRAS*, *MECOM/EVI1*, *MPL*, *MYC*, *NF1*, *NPM1*, *NRAS*, *PHF6*, *PPM1D*, *PRPF8*, *PTEN*, *PTPN11*, *RAD21*, *RIT1*, *RUNX1*, *SAMD9*, *SAMD9L*, *SETBP1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1* and *ZRSR2*. This gene panel has been validated on more than 1,000 BM samples with various myeloid neoplasms for the past 2 years (J. Soulier, personal communication). Libraries were sequenced (2 × 150 bp) on an MiSeq system (Illumina). Data analysis was performed using a homemade pipeline that included standard tools to identify point mutations, insertions or deletions, as well as copy number alterations. The pathogenic effect of the variants was assessed using guidelines from the American College of Medical Genetics and Genomics.

Array CGH analyses. Agilent 400K Array CGH technology was used in genomic BM DNA following the manufacturer's recommendations (Agilent Technologies). Arrays were scanned using SureScan High-Resolution Technology (Agilent Technologies), and the images were processed using Agilent Feature Extraction software, applying linear and lowest normalization methods and local background subtraction. Analysis was performed using the Agilent Genomic Workbench Version 7.0 software. The final profiles were validated via visual analysis by two investigators (A.R. and J.Soulier), considering the size and $\log_2$ratio of the deviation with respect to the individual background noise of each array at each particular chromosomal location.

Cell sorting of hematopoietic populations from PB and BM. Total BM and PB were obtained from patients at different time points post-infusion, as defined in the clinical protocol. Before the sorting of BM cells, erythrocytes were lysed with ammonium chloride lysis solution (0.155 mmol l^{-1} NH_4Cl + 0.01 mmol l^{-1} $KHCO_3$ + 10^{-4} mmol l^{-1} EDTA). Before the sorting of PB samples, erythrocytes were depleted with HES. Cells were then stained for 30 min at 4 °C, in the dark, using the monoclonal antibodies listed in Supplementary Table 1. DAPI was used at a concentration of 1 µg ml^{-1} as a viability marker.

Cells were sorted in a BD Influx (BD Biosciences) following the strategy shown in Supplementary Fig. 1. Offline analysis was performed with the FlowJo software. Cell pellets were stored at −80 °C for subsequent DNA extraction and quantitative PCR (qPCR) analysis.

Analysis of the lentiviral vector copy number. A total of 14 d after cell transduction, values of VCN cell^{-1} were determined, either in liquid cultures or in individual or pooled hematopoietic colonies grown in methylcellulose. After transplantation, values of VCN cell^{-1} were analyzed in PB and BM samples at different time points, after either HES fractionation or cell sorting. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), or by proteinase K lysis[36]. VCN cell^{-1} values were analyzed by duplex detection of the *Psi* sequence, normalized to *Albumin*, using primers specific for the *Psi* sequence (*Psi* forward (5′ CAGGACTCGGCTTGCTGAAG 3′) and *Psi* reverse (5′ TCCCCCGCTTAATACTGACG 3′)) and detected with the TaqMan probe (5′ CGCACGGCAAGAGGCGAGG3′). To normalize to endogenous *ALBUMIN*, specific primers for *ALBUMIN* were used (*Alb* forward (5′ GCTGTCATCTCTTGTGGGCTG 3′) and *Alb* reverse (5′ ACTCATGGGAGCTGCTGGTTC 3′), together with the TaqMan probe (5′ CCTGTCATGCCCACACAAATCTCTCC 3′). qPCR was conducted in an Applied Biosystems 7500 Fast Real Time PCR system (Thermo Fisher Scientific), as previously described[36].

ISA by standard linear amplification-mediated PCR (LAM-PCR) and deep sequencing. LAM-PCR was used to identify lentiviral vector flanking genomic sequences[37]. Briefly, 50–500 ng genomic DNA was used to amplify flanking sequences by two 50-cycle linear PCR amplification steps with biotinylated primers hybridizing to the 3′ region of the long terminal repeats of the vector. Subsequent steps involved magnetic capture of the biotinylated PCR products, hexanucleotide priming by Klenow polymerase for double-strand synthesis, and restriction digestion using MluCI and MseI. After digestion, a cohesive double-stranded MluCI- or MseI-specific adapter (linker cassette) including a molecular barcode was ligated to the restricted DNA, and one-fifth of the eluate was used as a template in an exponential PCR using a biotinylated vector- and adapter-specific primer. Magnetic capture of the biotinylated PCR products was performed before using one-tenth of the eluate for re-amplification by a second exponential PCR step with nested vector- and adapter-specific primers. Preparation for deep sequencing was performed as previously described[38,39] and adapted for the MiSeq instrument (Illumina). Therefore, an additional PCR with special fusion primers carrying MiSeq-specific sequencing adapters was performed. DNA barcoding was used to allow parallel sequencing of multiple samples in a single sequencing run.

Bioinformatics analyses of sequenced LAM-PCR amplicons. Raw sequence data were trimmed according to sequence quality (Phred 30). Only sequences carrying correct (100% sequence identity) barcodes in both molecular barcodes (linker cassette barcodes and sequencing barcodes) were further analyzed. Primary insertion-site annotation was conducted using GENE-IS (version 1.2)[40]. In brief, sequences were trimmed (vector- and linker-cassette-specific parts removed) and aligned to the human genome using UCSC BLAT (hg38), while nearby genes and other integrating features were annotated as previously described[39]. Only sequences that showed at least 18 nucleotides of vector-specific sequence after MiSeq-specific fusion-primer sequencing were analyzed further to ensure the analysis of specific PCR products and vector–genome junctions, respectively.

Statistical analysis. Data for CFCs represent mean ± s.e.m. values of colonies scored in three independent plates, seeded with either patients' BM cells or with transduced CD34$^+$ cells. Linear regression analysis was used to study the correlation between the survival of CFCs after MMC administration and the percentage of corrected CD34$^+$ cells corresponding to the same BM sample (n = 15 independent samples). Statistical analyses were performed using the GraphPad Prism software package for Windows (version 7; GraphPad Software).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Extended Data and Supplementary Information files.

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a)

Patient ID	Gender	Ethnia	Compl. Group	Mutations	Protein
FA-02002	Male	Gypsy	FA-A	c295C>T	Truncated protein, p.Gln99*
				c295C>T	Truncated protein, p.Gln99*
FA-02004	Male	Caucasic	FA-A	c.1115_1118delTTGG	p.V372AfsX42
				c.1115_1118delTTGG	p.V372AfsX42
FA-02005	Male	Gypsy	FA-A	c295C>T	Truncated protein, p.Gln99*
				c295C>T	Truncated protein, p.Gln99*
FA-02006	Male	Caucasic	FA-A	exon38:c.3788_3790del	p.Phe1263del
				exon29:c.2851C>G	p.Arg951Gly

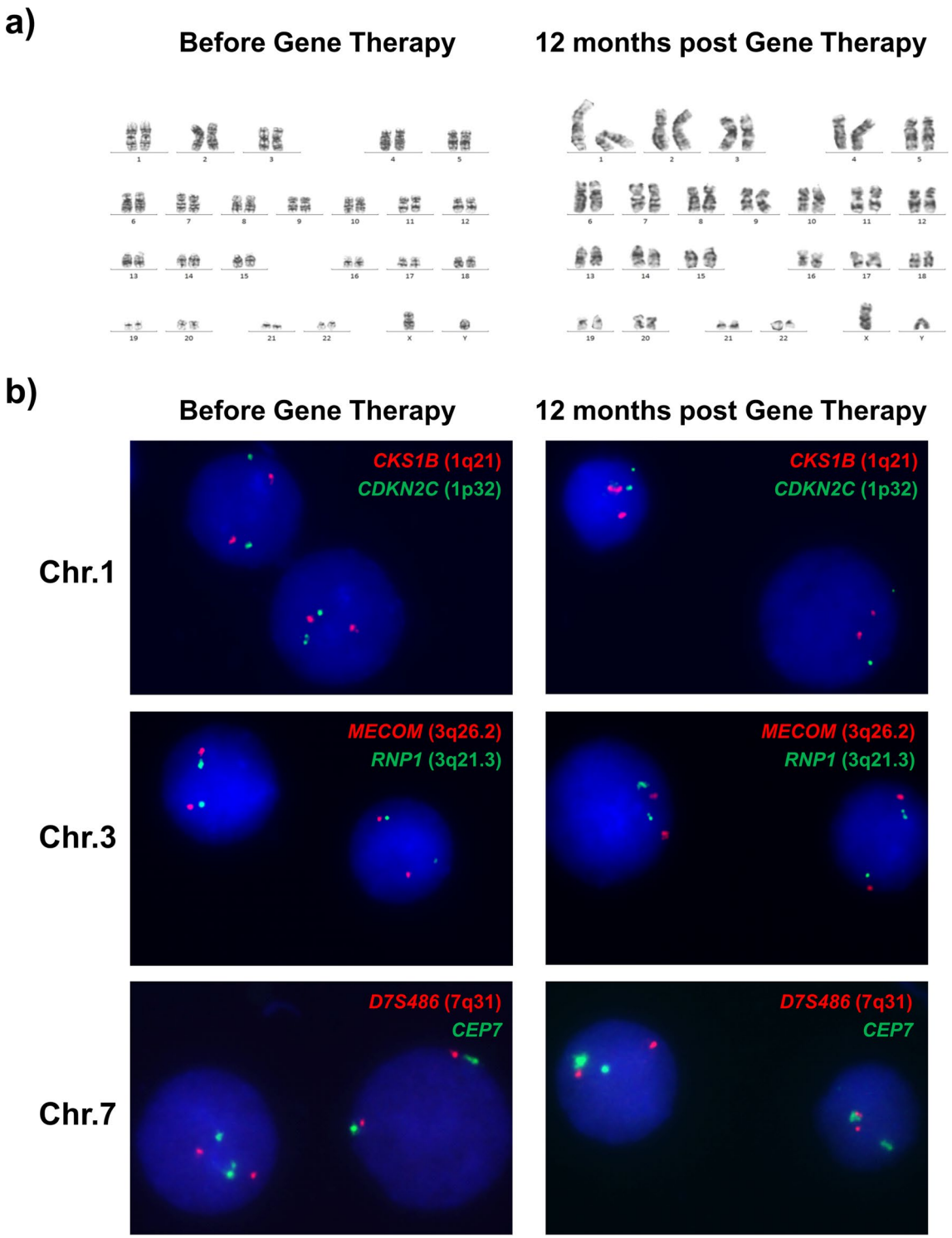
b)

Patient ID	Cryopr. $CD34^+$ cells	Screening visit	Age (years)	Hb (g/dL)	Neutroph/ µL	Platelets/ µL	BM $CD34^+$ cells/µL	BM $CD34^+/CD38^-$ cells/µL	CFCs/µL	CFCs Surv. to MMC (10 nM)	% aberrant T cells (DEB test)
FA-02002	Yes	HSC Collection	3.4	12.7	2,400	84,000	545.8	13.26	13.87	0.0 %	80%
		HSC Gene therapy	5.2	10.5	1,600	29,000	135.0	12.29	2.81	0.1 %	86%
FA-02004	Yes	HSC Collection	5.9	12.3	1,000	68,000	35.3	1.56	2.70	3.9 %	66%
		HSC Gene therapy	7.6	10.8	900	46,000	25.1	0.99	0.80	0.0 %	80%
FA-02005	No	HSC Collection/ Gene Therapy	4.0	12.5	1,680	38,000	276.1	0.39	5.25	0.0 %	74%
FA-02006	No	HSC Collection/ Gene Therapy	6.6	11.3	760	79,000	34.1	3.15	5.05	0.0 %	64%

Extended Data Fig. 1 | Characteristics of the patients with FA enrolled in the gene therapy trial. a,**b**, Genotype (**a**) and hematological and hematopoietic characteristics (**b**) of the recruited patients. In two patients (FA-02002 and FA-02004), $CD34^+$ cells were collected and then cryopreserved until PB cell counts decreased to levels defined in the gene therapy trial. At that time, cells were thawed, transduced and infused. PB cell counts corresponding to patients FA-02005 and FA-02006 fit the criteria required for both the collection and the infusion of transduced cells. In these two patients, cells were transduced and infused immediately after collection. The table shows PB cell counts and the total content of hematopoietic progenitors per microliter of BM. Additionally, CFC resistance to MMC and percentages of T cells with chromosomal breaks after DEB challenge are shown. CFC values represent mean values corresponding to the scoring of three plates seeded with BM cells obtained before HSC collection and before HSC gene therapy. A total of 25–50 metaphases were analyzed in the DEB tests.

PATIENT	$CD34^+$ cells/ kg	% $CD34^+$ cells	CFCs/kg	Mean VCN/cell (Total colonies)	CFCs Survival to MMC (10 nM)	Transduced $CD34^+$cells/kg	Transduced CFCs/kg
FA 02002 (Cryopr)	$0.55x10^6$	90.6%	$3.2x10^4 \pm 6.5x10^3$	$0.45^{\#}$	30.3%	$2.5x10^5$	$1.4x10^4 \pm 2.9x10^3$
FA 02004 (Cryopr)	$0.71x10^6$	57.0%	$3.2x10^4 \pm 3.8x10^3$	$0.24^{\$}$	27.0%	$1.6x10^5$	$7.3x10^3 \pm 8.8x10^2$
FA 02005 (Fresh)	$1.30x10^6$	80.5%	$1.7x10^4 \pm 2.4x10^3$	$0.17^{\#}$	12.1%	$2.2x10^5$	$2.8x10^3 \pm 4.0x10^2$
FA 02006* (Fresh)	$0.77x10^6$	3.5%	$3.1x10^5 \pm 2.2x10^4$	$0.53^{\#}$	49.2%	$4.1x10^5$	$1.6\ x10^5 \pm 1.2x10^4$

Extended Data Fig. 2 | Characteristics of cell products transduced with the therapeutic PGK-FANCA-WPRE* lentiviral vector and infused in non-conditioned patients with FA-A. G-CSF and plerixafor mobilized $CD34^+$ cells were pre-stimulated for 8–10 h and then transduced with the therapeutic lentiviral vector for another 12–14 h, as described in the Methods. Values of VCN $cell^{-1}$ were determined in hematopoietic colonies after 14 d of growth in semisolid cultures. In this case, determinations were carried out either in pooled (marked by a hashtag) or individual colonies (marked by a dollar sign). In no instance were mean VCN $cell^{-1}$ values in lentiviral vector-positive colonies higher than 1.0 copies $cell^{-1}$. Therefore analyses of the proportion of corrected cells in the PB and BM of treated patients (Fig. 1) are based on the assumption of the presence of 1.0 VCN in transduced cells. Cell viabilities in the infused products, including cryopreserved products, ranged between 84 and 97%. An asterisk denotes that $CD34^+$ cells from one of the two apheresis procedures were purified using a modified immunoselection procedure used to improve the yield of $CD34^+$ cells (see Methods). CFC values represent means ± s.e.m., corresponding to the scoring of three plates seeded with cells from each transduced product.

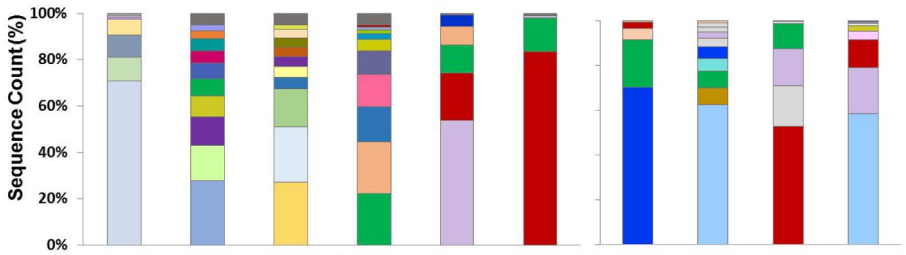


Extended Data Fig. 3 | Representative cytogenetic analysis of BM cells from gene therapy-treated patients with FA-A. a,**b**, Representative G-banding karyotypes (**a**) and FISH analyses of chromosomes 1q, 3q and 7q (**b**) of BM samples obtained before and after infusion of transduced cells. For cytogenetic studies, 10–20 metaphases of BM cells obtained before and at different time points after the infusion of transduced cells were examined. In total, 200 interphase nuclei per FISH were scored.

FA-PATIENT	Monts post-infusion	CYTOGENETICS	FISH Chr1	FISH Chr3	FISH Chr7	ArrayCGH
FA-02002	0	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
	6	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
	12	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
	24	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
FA-02004	0	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
	6	No Metaphases	NORMAL	NORMAL	NORMAL	No Abnormalities
	12	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
FA-02005	0	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
	6	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
	12	No Metaphases	NORMAL	NORMAL	NORMAL	No Abnormalities
	24	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
FA-02006	0	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
	6	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities
	12	46,XY	NORMAL	NORMAL	NORMAL	No Abnormalities

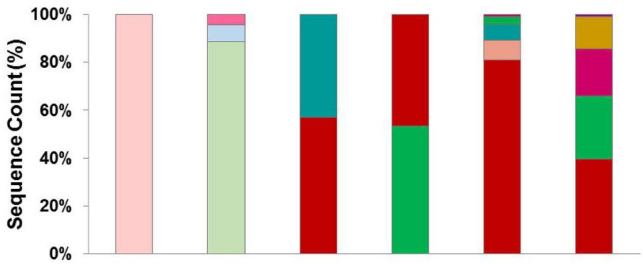
Extended Data Fig. 4 | Cytogenetic and high-throughput sequencing studies in BM cells from patients with FA-A treated by gene therapy. The table shows G-banding karyotypes, FISH analyses of chromosomes 1q, 3q and 7q, and array CGH analyses using 400K arrays (Agilent Technologies) of BM samples obtained before and at different times after infusion of transduced cells. The table shows that no cytogenetic nor genomic abnormalities were observed in any of the treated patients, either before or after the infusion of transduced HSCs. Cytogenetic studies were conducted in 10–20 metaphases. In total, 200 interphase nuclei per FISH were scored.

FA 02004



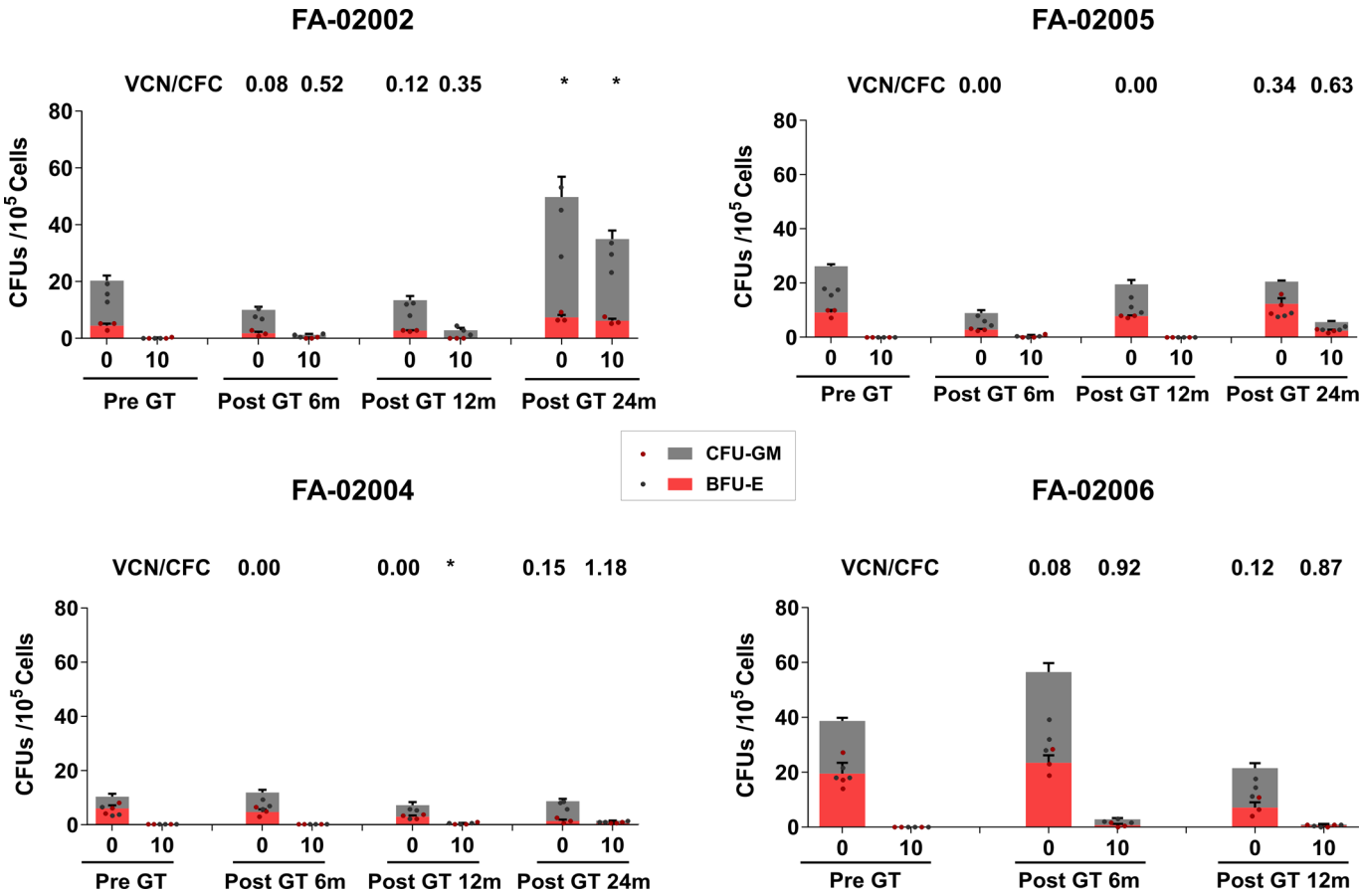
Subject	FA-02004						FA-02004			
Cell Type	PBMCs						CD14	CD15	CD19	CD3
Time Point	4W	4M	6M	9M	12M	18M	24M			
Rank										
Top1	CTCF	MECOM	PDE4D	EP400	ZFAND3	B3GNT3	BRCA1	ZMYM2	B3GNT3	ZMYM2
Top2	ODF3B	NACC2	IFT140	ZCCHC14	B3GNT3	EP400	EP400	ERN1	SPNS3	ZFAND3
Top3	PACS1	SPNS3	NUP62CL	RIC1	EP400	ZFAND3	ZCCHC14	EP400	ZFAND3	B3GNT3
Top4	AFMID	YLPM1	RIC1	PSMD11	ZCCHC14	GNG2	B3GNT3	MALAT1	EP400	PDIK1L
Top5	EEF1DP3	EP400	SUMO1P1	EMCN	BRCA1	ERN1	UNK	BRCA1	B3GNT3	PRKACB
Top6	GNG7	LRRC16A	SPNS3	MIR1268A	MALAT1	STAT3	CCDC47	CCDC47	ZCCHC14	ARFGAP3
Top7	STX8	GPATCH8	UBE2G1	EIF4G3	ITCH	ZMYM2	PDIK1L	ZFAND3	XPO6	ITCH
Top8	MARCH2	ATR	TBC1D22B	SKAP1	KAT5	ITCH		ABCA10	DLEU2	ZFAND3
Top9	ZNF791	PDE8B	FOXJ3	ITCH	KAT5	ITCH		SLC37A1	B3GNT3	ITCH
Top10	SUSD6	RELA	QSER1	B3GNT3		B3GNT3		ZCCHC14	B3GNT3	MYO1D
UIS	14	56	25	29	9	24	7	24	19	32

FA 02005



Subject	FA-02005					
Cell Type	PBMCs					
Time Point	4W	6M	12M	18M	24M	30M
Rank						
Top1	NFYC	ATAD3B	RNF38	DAZAP1	RNF38	RNF38
Top2		SLA	TET2	RNF38	PIK3C2A	DAZAP1
Top3		FAIM3	SLC37A1	MAP1S	TET2	STIP1
Top4		SLC37A1	TMED5		DAZAP1	RUVBL2
Top5		C3orf22			STIP1	RUVBL2
Top6		LINC00887			MAP1S	MAP1S
Top7		LAMC3			RNF38	SMARCA2
Top8					PIK3C2A	MAP1S
Top9					MLKL	DAZAP1
Top10					UPRT	MAP1S
UIS	1	7	4	3	10	17

Extended Data Fig. 5 | Genome-wide mapping of vector UISs in PB cells of the patients FA-02004 and FA-02005 after gene therapy. The ten most represented UISs in PB cells from the patients FA-02004 and FA-02005 at different times after gene therapy are shown. Samples were analyzed as in Fig. 3. UISs detected at more than one time point, or with a contribution higher than 1% at one time point are marked with individualized colors. UISs marked in dark gray are those not represented in the top ten rank. RefSeq genes nearest to the UISs are listed in the table. The top ten clones marked in light gray indicate clones with a contribution lower than 1%. The total number of UISs is indicated at the bottom of each column. Because of the high contribution of clone B3GNT3 in PB cells from patient FA-02004 at 18 months post-infusion, analyses carried out at 24 months were conducted in sorted cell lineages, which revealed a marked reduction in the representation of this clone.



Extended Data Fig. 6 | Hematopoietic progenitor content in patients with FA-A before and after gene therapy. Analysis of the CFC content in BM cells from patients with FA before and after infusion of transduced CD34⁺ cells. Numbers of hematopoietic colonies generated in the presence and absence of MMC (10 nM) are presented. Individual data are shown in dot plots and mean values are represented by bars. Ratios between these numbers indicate the relative MMC resistance of CFCs at different times post-infusion (data shown in Fig. 3). CFC values represent means ± s.e.m. corresponding to the scoring of three plates seeded with BM cells obtained before and after infusion of transduced CD34⁺ cells. Vector copy numbers per cell obtained in hematopoietic colonies (CVN/CFC) are also represented. An asterisk represents samples not evaluable since the quantity of DNA was under the detection limit for amplification of the human albumin gene by qPCR.

nature research

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Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	CD34+ cells were immunoselected with the Clini-MACS device, using the software version 2.4., and the selection program 1.2 or 3.1; VCN was analyzed using Applied Biosystems® 7500 Real-Time PCR Systems; Immune response to FANCA was measured with the ELISPOT reader (AID Autoimmun Diagnostika GmbH);Chromosomal aberrations were analyzed using Zeiss Imager M1 microscope; FANCD2 foci were studied with a microscope Axioplan 2 imaging (Carl Zeiss) and AxioCam MRm camera(Carl Zeiss); Targeted gene sequencing libraries were prepared by using SureSelect XT Target Enrichment (Agilent Technologies); Libraries were sequenced in a MiSeq system (Illumina Inc); Array CGH was conducted using Agilent 400K Array-CGH technology and scanned with SureScan High-Resolution Technology (Agilent Technologies); Cell sorting was conducted in a BD INFLUX™ (BD Biosciences);
Data analysis	Flow cytometry data was analyzed by FlowJo™ software LLC. Metaphase finder (Metasystems) was used to study chromosomal breaks and FISH. AxioVision 4.6.3 (Carl Zeiss) and Corel Photo-Paint 11 (Corel) were used to process FANC2 foci images. Array CGH assays were analized by Agilent Genomic Workbench Version 7.0. Primary IS annotation was conducted by using GENE-IS (version 1.2). Graphical ouput and statistical analysis were performed with GraphPad Prism software package for Windows (version 7, GraphPad Software).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The authors declare that data supporting the findings of this study are available within the paper and its Extended data and Supplementary information files.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	This gene therapy clinical trial is planned to enroll 10 patients in two cohorts. Data presented in this study represents the first cohort of 4 patients. Sample size is based on feasibility.
Data exclusions	Technically validated results were always included to the analyses. We did not apply any exclusion criteria for outliers
Replication	Colony forming cell assays were carried out by triplicate. Data are represented as mean values +- SEM. For VCN determinations, qPCR was validated for reproducibility. For total PB at least two independent determinations were done with each sample. In purified PB and BM subpopulations with limited amounts of DNA, single VCN determinations could be done in some instances, always in parallel with control DNA samples harboring known copies of the provirus. In all qPCR analyses a standard curve with one or two positive controls with 1 or 3 copies per cell were used. Nuclear FANCD2 foci: Analyses were done in 200 cells per slide. Insertion site analyses represent individual analyses of PB samples obtained at different time points post-infusion. 2,001 unique integration sites were identified in the four treated patients. Chromosomal instablity tests: Individual analyses of 25-50 metaphases were carried out at different time points after the infusion of transduced cells. Cytogenetic Analyses: Individual analyses of 10-20 metaphases were conducted at different time points after the infusion of transduced cells. FISH: Two hundred interphase nuclei per FISH were scored.
Randomization	This is a non randomized trial
Blinding	The experiment design did not include allocation to groups or blinding. However, data was acquired based only on identification numbers.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Antibody against human FANCD2 (ab2187-50, Abcam) and secondary antibody IgG against rabbit, conjugated with AlexaFluor 594 (ab150080, Abcam; 1:1,000 dilution); CD45 APC, CD34 PE and CD38 antibodies (all from Beckton Dickinson); CD14 FITC RM052 Beckman Coulter; CD15 PE 80H5 Beckman Coulter; CD3 APC UCHT1 Biolegend; CD19 PECY5 HIB19 Biolegend; CD71 PE YDJ1.2.2. Beckman Coulter; CD235a FITC 11E4B-7-6 Beckman Coulter CD34 PECY7 4H11 eBiosciences; CD45 APC 2D1 Biolegend; CD41 PE P2 Beckman Coulter; CD42B FITC H1P1 Becton Dickinson
Validation	FANCD2 and AlexaFluor 594 were tested previously in Healthy donor and FA-A patient cell lines(Diez et al., 2017). Similar data was also confirmed in Abcam webpage. All the antibodies used for flow cytometry (CD45 APC;CD34 PE; CD38 FITC; CD14 FITC ; CD15 PE ; CD3 APC; CD19 PECY5 CD71 PE; CD235a FITC; CD34 PECY7; CD45 APC; CD41 PE; CD42B FITC) belong to commercial brands that ensure quality control management of their products (https://www.beckmancoulter.com/es, https://www.bd.com/, https://www.thermofisher.com/es/es/home/life-science/antibodies/ebioscience.html, https://www.biolegend.com/). All the clones have been assigned to their CD during the HLDA Workshop on Human Leucocyte Differentiation Antigens (http://www.hcdm.org/). Flow cytometry CD34+ determinations are based on ISHAGE (Sutherland et al., 1996) guidelines, which include selection of

bright fluorochrome, vital nucleic acid dye (to exclude platelets, unlysed red cells, and debris) or use of 7AAD (to exclude dead cells), counterstaining with CD45 mAb, boolean gating to resolve the CD34+ (low levels of CD45 expression and low SSC), inclusion of CD34dim and CD34bright populations, omission of the negative control staining and when possible at least 100 CD34 + cells to ensure a 10% precision.
For the sorting of mature populations and to achieve accurate results, gating of all the markers vs SSC was performed to ensure the main phenotype for each marker (see gating strategies). For all cell sortings, additional factors where considered such as exclusion of debris, exclusion of cell doublets, appropriate fluorophore brightness, negative controls when needed, exclusion of non-viable cells and staining with a shared marker (i.e. CD45 leukocyte marker) when necessary.
References:
Diez, B., Genovese, P., Roman-Rodriguez, F.J., Alvarez, L., Schiroli, G., Ugalde, L., Rodriguez-Perales, S., Sevilla, J., Diaz de Heredia, C., Holmes, M.C., et al. (2017). Therapeutic gene editing in CD34(+) hematopoietic progenitors from Fanconi anemia patients. EMBO molecular medicine 9, 1574-1588.
Sutherland DR1, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother. 1996 Jun;5(3):213-26.

Human research participants

Policy information about studies involving human research participants

Population characteristics	Eligible patients were Fanconi anemia patients, subtype A. Detailed characteristics of treated patients are shown in Methods and in Extended Data 1.
Recruitment	Participants were referred by specialized centers on Fanconi anemia without any self-selection of candidates. Patients were screened for inclusion and exclusion criteria. Subjects meeting all inclusion criteria and without any exclusion criteria were enrolled. Written informed consent was obtained from the parents.
Ethics oversight	The study was approved by the Ethic Committees of Hospital Hospital Infantil Universitario Niño Jesús (Madrid) and Hospital Universitari Vall d'Hebron (Barcelona)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	For the collection of mobilized HSCs, patients were included in the FANCOSTEM clinical trial protocol (NCT02931071; EudraCT 2011-006197-88). For gene therapy, patients were included in the FANCOLEN-1 trial (NCT 03157804; EudraCT 2011-006100-12).
Study protocol	The most relevant information of the clinical trial is shown in the summary included in the registration of the trial (NCT 03157804; EudraCT 2011-006100-12). The full trial protocol can be provided upon request to the PI of the clinical trial, Julian Sevilla, at julian.sevilla@salud.madrid.org
Data collection	Data are collected at Hospital Val d'Hebron in Barcelona and Hospital del Niño Jesús in Madrid. All data collection are supervised periodically by the CRO, Alphabioresearch.
Outcomes	Since this trial does not imply the conditioning of the patients, and given that there were not previous data revealing engraftment of gene corrected cells, the primary outcome was defined based on the vector copy number per cell in peripheral blood, as determined by qPCR analysis. The secondary outcome was based on the hematological analysis of the patients.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Immunophenotypic analyses of HSPCs were conducted by flow cytometry using anti-CD45 APC, CD34 PE and CD38 FITC antibodies (all from Beckton Dickinson). Fluorochrome-matched isotypes were used as controls. 4',6-Diamidino-2-phenylindole (DAPI; Roche)-positive cells were excluded from the analysis. Analysis was performed using FlowJo™ software LLC . For cell sorting analysis total BM and PB were obtained from patients at different time points post-infusion, as defined in the clinical protocol. Prior to the sorting of BM cells, erythrocytes were lysed with ammonium chloride lysis solution (0.155 mmol/L NH4Cl + 0.01 mmol/L KHCO3 + 10−4 mmol/L EDTA). Prior to the sorting of PB samples, erythrocytes were depleted with hydroxy-ethyl starch (HES). Cells were then stained for 30 min at 4ºC, on dark, by using the moAbs listed here:

	CD14 FITC; CD15 PE; CD3 APC; CD19 PECY5; CD71 PE; CD235a FITC; CD34 PECY7; CD45 APC; CD38 FITC; CD41 PE; CD42B FITC DAPI was used at a concentration of 1 µg/mL as viability marker.
Instrument	Flow cytometry analysis were conducted using LSR Fortessa (Becton Dickinson Pharmingen) Cells were sorted in a BD INFLUX™ (BD Biosciences).
Software	FlowJo™ software LLC
Cell population abundance	Sorting was perfomed in four a way Yield mode to ensure recovery of all possible events. The abundance of the relevant cell populations, determined by testing the sorted cells again by FACS, reached >90%. FACS data were analyzed by FlowJo v10.
Gating strategy	In all Flow Cytometry and Cell sorting procedures performed in this study, populations were first selected based on their FSC-A/ SSC-A. Then doublets were excluded by representing Trigger Pulse/ Height/Weight parameter vs Area depending on the equip. Dead cells were excluded by DAPI staining positive in all cases. Specifically for the immunophenotyping of CD34+ both gating and percentages where calculated by its negative of fluorescence minus one controls (FMO) in all samples. Different cell populations were isolated by FACS, including CD3+ T cells, CD19+ B cells, CD14+ Monocytes and CD15+ Granulocytes. Cells expressing these markers were gated based on the respective negative controls of unstained cells, simple positive controls and SSC-A/FSC-A profiles to ensure the population.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.