Gene therapy restores the transcriptional program of hematopoietic stem cells in Fanconi anemia

Miren Lasaga,[1*] Paula Río,[2,3,4*] Amaia Vilas-Zornoza,[5,6*] Nuria Planell,[1] Susana Navarro,[2,3,4] Diego Alignani,[7] Beatriz Fernández-Varas,[3,8] Daniel Mouzo,[1] Josune Zubicaray,[9] Roser M. Pujol,[3,10,11] Eileen Nicoletti,[12] Jonathan D. Schwartz,[12] Julián Sevilla,[3,9] Marina Ainciburi,[5,6] Asier Ullate-Agote,[5,6] Jordi Surrallés,[3,10,11] Rosario Perona,[3,8,13] Leandro Sastre,[3,8] Felipe Prosper,[5,6#] David Gomez-Cabrero,[1,14,15#] and Juan A. Bueren[2,3,4#]

[1]Translational Bioinformatics Unit, Navarrabiomed, Universidad Pública de Navarra (UPNA), IdiSNA, Pamplona, Spain; [2]Hematopoietic Innovative Therapies Division, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain; [3]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain; [4]Instituto de Investigaciones Sanitarias, Fundación Jiménez Díaz, Madrid, Spain; [5]Area de Hemato-Oncología, Centro de Investigación Médica Aplicada (CIMA), and Servicio de Hematologia y Terapia Celular, Clínica Universidad de Navarra, IDISNA, Pamplona, Spain; [6]Centro de Investigación Biomédica en Red de Cáncer, CIBERONC, Madrid, Spain; [7]Flow Cytometry Core, CIMA, Universidad de Navarra, Pamplona, Spain; [8]Instituto de Investigaciones Biomédicas Alberto Sols, CSIC/UAM, Madrid, Spain; [9]Hemoterapia y Hematología Pediátrica, Fundación para la Investigación Biomédica, Hospital Infantil Universitario Niño Jesús, Madrid, Spain; [10]Departmento de Genética y Microbiología, Universitat Autónoma de Barcelona, Barcelona, Spain; [11]Fundación Instituto de Investigación del Hospital de la Santa Creu y Sant Pau, Barcelona, Spain; [12]Rocket Pharmaceuticals Inc., New York, NY, USA; [13]Instituto de Salud Carlos III, Madrid, Spain; [14]Biological and Environmental Science and Engineering Division, King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia and [15]Bioscience Program, King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia

ML, PR and AV-Z contributed equally as first authors.

#JAB, DG-C and FP contributed equally as senior authors.

Correspondence: Juan A. Bueren
juan.bueren@ciemat.es

David Gomez-Cabrero
david.gomezcabrero@kaust.edu.sa

Felipe Prosper
fprosper@unav.es

Received: November 16, 2022.
Accepted: March 29, 2023.
Early view: April 6, 2023.

https://doi.org/10.3324/haematol.2022.282418

Abstract

Clinical trials have shown that lentiviral-mediated gene therapy can ameliorate bone marrow failure (BMF) in non-conditioned Fanconi anemia (FA) patients resulting from the proliferative advantage of corrected FA hematopoietic stem and progenitor cells (HSPC). However, it is not yet known if gene therapy can revert affected molecular pathways in diseased HSPC. Single-cell RNA sequencing was performed in chimeric populations of corrected and uncorrected HSPC co-existing in the BM of gene therapy-treated FA patients. Our study demonstrates that gene therapy reverts the transcriptional signature of FA HSPC, which then resemble the transcriptional program of healthy donor HSPC. This includes a down-regulated expression of TGF-β and p21, typically up-regulated in FA HSPC, and upregulation of DNA damage response and telomere maintenance pathways. Our results show for the first time the potential of gene therapy to rescue defects in the HSPC transcriptional program from patients with inherited diseases; in this case, in FA characterized by BMF and cancer predisposition.

Introduction

The potential of gene therapy (GT) to correct a variety of human inherited diseases, including primary immunodeficiencies and hemoglobinopathies, has been well demonstrated in previous clinical studies.[1] In addition, we have recently shown that lentiviral-mediated GT can also enable correction of more complex diseases, such as Fanconi anemia (FA), in which marked phenotypic defects are already evident in the self-renewing hematopoietic stem cells (HSC). As previously reported, the infusion of corrected CD34$^+$ cells in non-conditioned FA patients resulted in a marked hematopoietic stem and progenitor cell (HSPC) proliferative advantage, which facilitates the progressive increase in the number of gene-corrected cells in the bone marrow (BM) and peripheral blood (PB).[2]

Despite significant advances in the field of GT, the full potential of this therapeutic modality will depend on its capacity to re-establish the molecular circuits and the functional potential of diseased cells. This is even more rel-

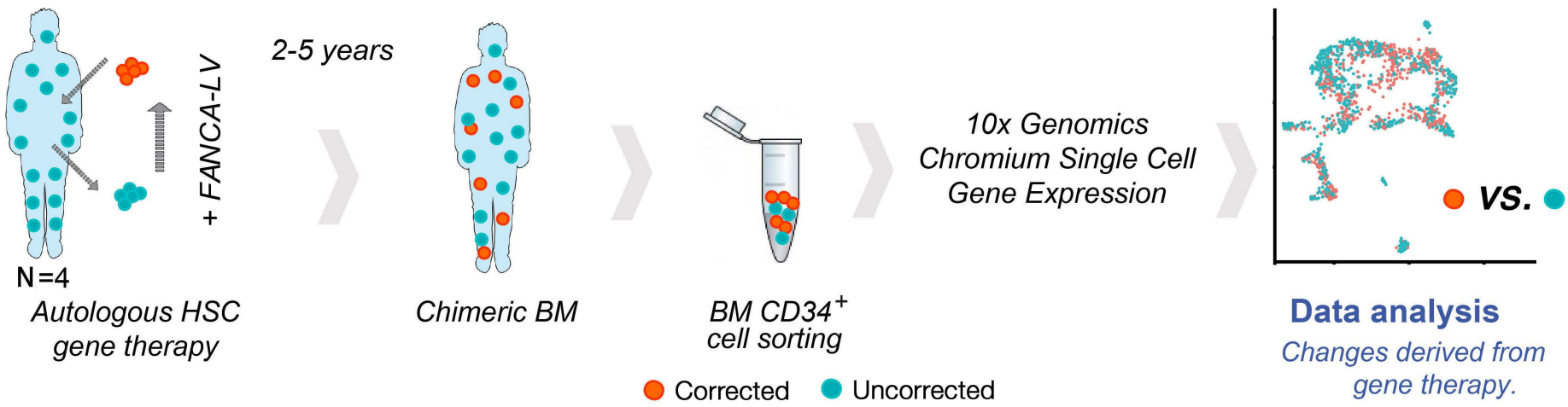


Figure 1. Experimental design of scRNAseq analyses performed in Fanconi anemia group A patients 2-5 years after lentiviral-mediated gene therapy. Four Fanconi anemia group A (FA-A) patients who had been treated with *ex vivo* lentiviral-mediated gene therapy in the absence of conditioning were included in this study. At 2-5 years post gene therapy, these patients harbored a chimeric population of corrected and uncorrected hematopoietic stem and progenitor cells (HSPC) in their bone marrow (BM). Aliquots of BM CD34$^+$ cells from these patients were sorted and processed for single-cell RNA-seq. Bioinformatic analyses comparatively investigated changes in the transcriptional program of corrected *versus* uncorrected HSPC, co-existing in each of the gene therapy-treated patients. HSC: hematopoietic stem cell.

evant in syndromes such as FA, characterized by DNA repair defects resulting in progressive accumulation of DNA damage and additional molecular responses triggered by a defective FA/BRCA pathway. In patients treated in the FANCOLEN-I clinical trial, the presence of a chimeric population of corrected and uncorrected FA HSPC, none of which had been exposed to any conditioning agent, has allowed us to comparatively investigate the differential molecular pathways that characterize each of these populations co-existing in the BM of these patients (Figure 1). This approach allowed us to demonstrate that lentiviral-mediated GT not only mediates a progressive engraftment of gene-corrected HSPC in FA patients, but also results in the reprogramming of the transcriptional signature of FA HSPC, which then resembles those of healthy HSPC, thus accounting for the phenotypic correction of FA HSPC.

Methods

Patients and healthy donors

Fanconi anemia patients included in this study (patients FA-02002, FA-02004, FA-02006 and FA-02008) were Fanconi anemia group A (FA-A) patients enrolled in the FANCOLEN-1 gene therapy trial (FANCOLEN-1; clinicaltrials.gov NCT03157804; European Clinical Trials Database 2011-006100-12) and were 5, 7, 6 and 3 years old, respectively, at the time of transduced cell infusion. Patients complied with all relevant ethical regulations approved by the ethics committees at the Hospital Vall d'Hebron in Barcelona and the Hospital del Niño Jesús in Madrid.

Patients were infused with autologous CD34$^+$ cells after transduction with the therapeutic PGK-*FANCA*.Wpre* LV.[3] Recent clinical data have shown that although no conditioning was given to these patients prior to cell infusion, a progressive engraftment of corrected cells took place over time, implying the presence of a chimeric population of corrected and uncorrected cells in their hematopoietic tissues.[2] For the participation of healthy donors (HD) in this study (median age 20 years), BM aspiration was performed after informed consent. The study was approved by the clinical research ethics committee of the Clínica Universidad de Navarra.

Bone marrow cells

Bone marrow cells from GT-treated patients were obtained in the course of routine follow-up studies of the GT trial and as part of additional exploratory studies. Samples used in these studies were obtained 5 years (FA-02002), 4 years (FA-02004), 3 years (FA-02006), and 2 years (FA-02008) after infusion of transduced CD34$^+$ cells, respectively. Patients FA-02004 and FA-02008 had been treated with eltrombopag to stimulate hematopoiesis 12 and 6 months prior to the evaluation of BM cells, respectively. All samples were processed immediately after BM aspiration.

For the purification of CD34$^+$ cells, erythrocytes were lysed with ammonium chloride lysis solution (0.155 mmol/L NH_4Cl + 0.01 mmol/L $KHCO_3$ + 10^{-4} mmol/L EDTA), washed using PBS (Gibco) + 0.2%BSA (10%) + 2% PenStrep (Gibco) and stained using CD45 APC (clone 2D1; Biolegend) and CD34 PECY7 (clone 4H11; eBiosciences) for 30 minutes at 4°C. DAPI was used at a concentration of 1 μg/mL as a viability marker. CD34$^+$ cells were then sorted in a BD INFLUX™ (BD Biosciences) or BD FACSAria II (BD Biosciencs), as previously shown.[2] Purified CD34$^+$ cells were directly used for scRNA seq analysis and small aliquots stored at -80°C for vector copy number analysis.

Chromosomal instability test in peripheral blood T cells

The diepoxybutane (DEB)-induced chromosomal instabil-

ity test was carried out in PB T cells from the FA patients prior to and in the long-term after infusion of corrected cells. After 24 hours (h) of culture, PB cells were incubated in the absence or the presence of DEB (0.1 μg/mL; Sigma-Aldrich), and 46 h later colcemid was added (Gibco; 0.1 μg/mL). A further two hours later, metaphase spreads were obtained and stained with Giemsa. Fifty metaphases were analyzed from DEB-treated and 25 metaphases from unexposed cultures in a Zeiss Imager M1 microscope coupled to a computer-assisted metaphase finder (Metasystems). Criteria for the determination and quantification of chromosome breakage have been described previously.[4]

Additional information on patients and study methods is available in the *Online Supplementary Appendix*.

Results

Gene therapy modifies the hematopoietic stem and progenitor cell transcriptional program in Fanconi anemia patients

Bone marrow CD34$^+$ cells from four FA-A patients (patients FA-02002, FA-02004, FA-02006, and FA-02008) who had respectively been treated by gene therapy (GT) 5, 4, 3, and 2 years previously, were purified and analyzed by single-cell RNA sequencing (scRNAseq) (Figure 1). Quantitative polymerase chain reaction (qPCR) analyses from these samples showed the presence of 0.77 vector copy numbers (VCN) per cell in the BM of patient 02002, and 0.45, 0.26, and 0.29 VCN per cell in purified CD34$^+$ cells in patients FA-02004, FA-02006, and FA-02008, respectively (see Methods). Since the average VCN per corrected cell was of 1.0 copies,[2] the proportion of corrected cells in these patients is estimated to be 77%, 45%, 26%, and 29%, respectively, revealing the chimeric nature of the HSPC populations present in their BM.

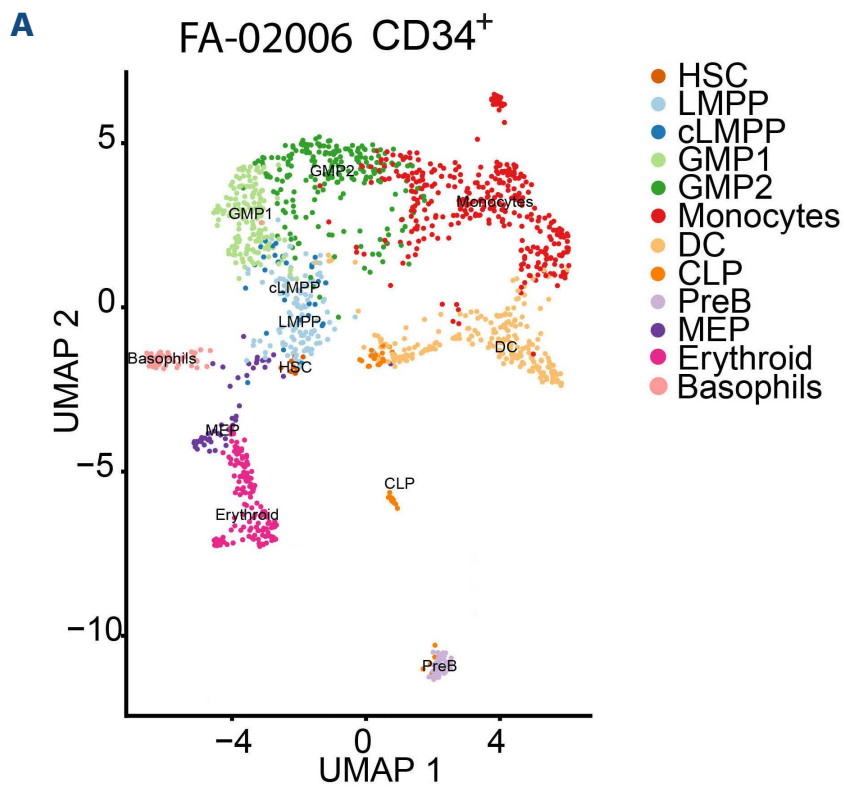
CD34$^+$ cells from FA patients were classified according to transcriptional profiles described previously,[5,6] which identified 12 different HSPC clusters that corresponded to primitive HSC and more committed lympho-hematopoietic progenitor cells. (See analyses from patient FA-02006 in Figure 2A, and patients FA-02002, FA-02004 and FA-02008 in *Online Supplementary Figure S1A-D*). To investigate the impact that GT had in the transcriptional program of FA HSPC, CD34$^+$ cell subpopulations from GT-treated FA patients were classified as FANCA$^+$ and FANCA$^-$, based on the expression of *FANCA* by scRNAseq (see Methods). Importantly, we observed that cells with reads mapping to the poly-A sequence of the viral vector, a region only present in gene therapy-treated cells, were enriched for FANCA$^+$ cells (P<0.001 for all patients). As a result, despite the sparsity of single-cell data, we concluded that gene-corrected cells are enriched in the FANCA$^+$ set.

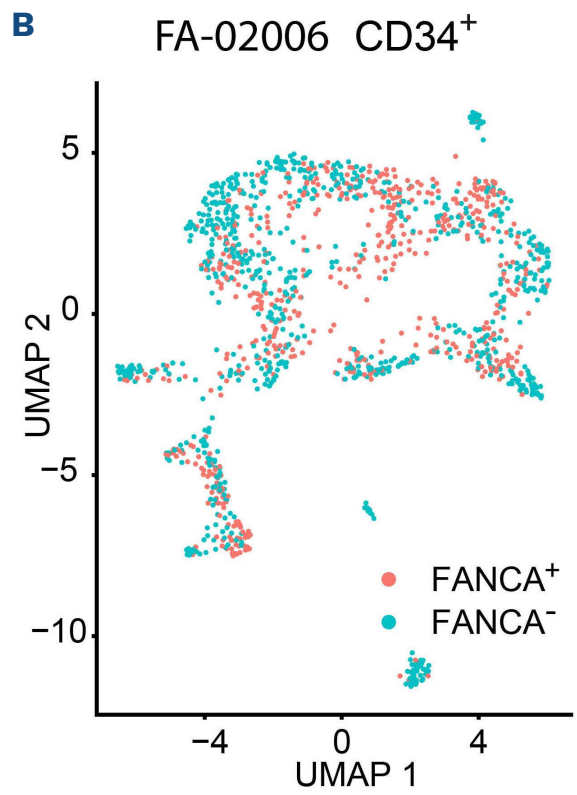
Next, we observed that, in most of the patients, the presence of FANCA$^+$ cells was evident in all HSPC populations (Figure 2B, C; see details for low prevalent cells in *Online Supplementary Figure S1E*). No significant differences in the proportion of FANCA$^+$ cells present in these subpopulations were observed among the four patients (*Online Supplementary Table S1*). In addition, a wide range of FANCA expression was observed in the samples analyzed, although higher expression levels were observed in most HSPC subpopulations from patient FA02002 compared with the other patients (Figure 2D).

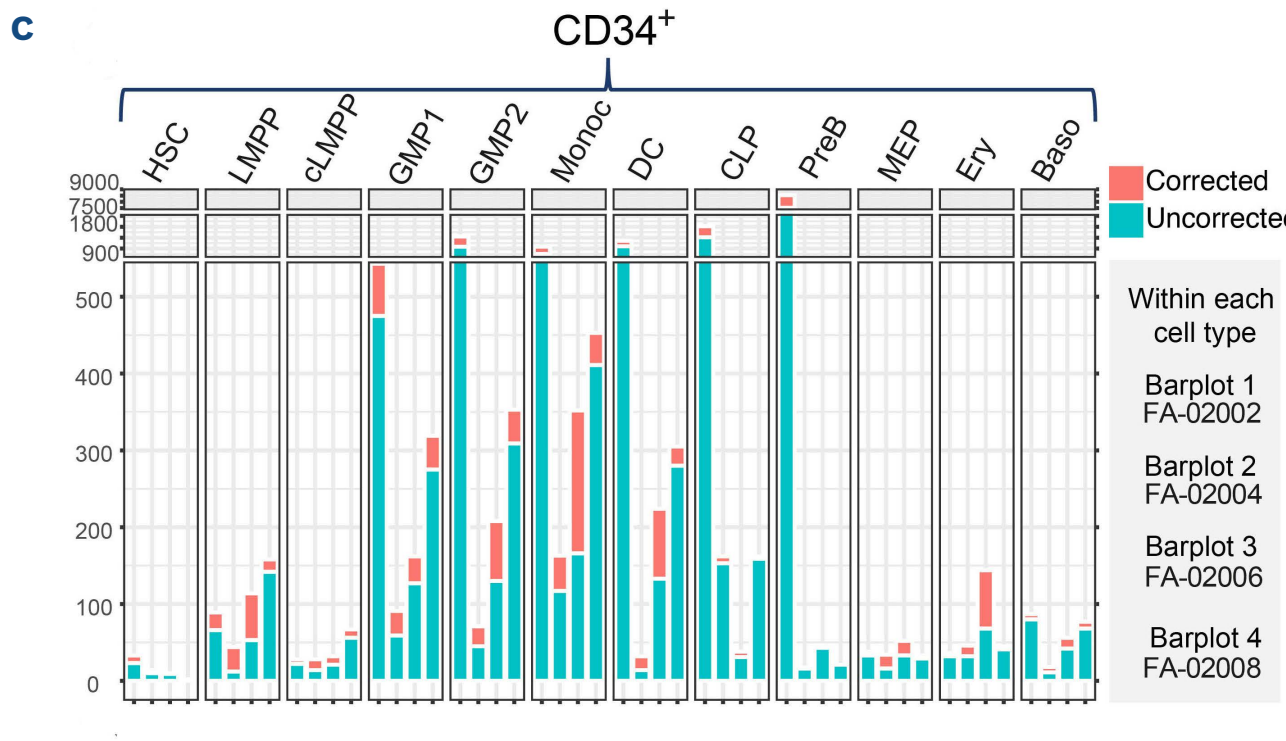
To investigate the impact that ectopic FANCA expression had in the transcriptional program of HSPC, we performed a differential expression analysis between FANCA$^+$ and FANCA$^-$ HSPC present in each GT-treated patient. The ectopic expression of *FANCA* up-regulated a high number of genes compared to the number of genes that were down-regulated (Figure 2E). As expected, genes with statistically differential expression corresponded to subpopulations with a higher representation (i.e., CD34$^+$ cells committed to the monocyte lineage). Genes with significantly up-regulated and down-regulated expression (P<0.001) are shown in Figure 2E. Remarkably, most of the up-regulated and down-regulated genes in FANCA$^+$ *versus* FANCA$^-$ cells showed the same expression pattern in each of the analyzed patients (Figure 2E). To select the genes with a robust differential expression between FANCA$^+$ *versus* FANCA$^-$ HSPC, we considered those genes that showed a significant differential expression (abs(logFC)>0.25 and adjusted P-value <0.05) in at least one cell type and in at least three patients. In addition, changes in gene expression should be in the same direction in all the four patients. Based on these criteria, a total number of 152 differentially expressed genes were identified (*Online Supplementary Table S2*).

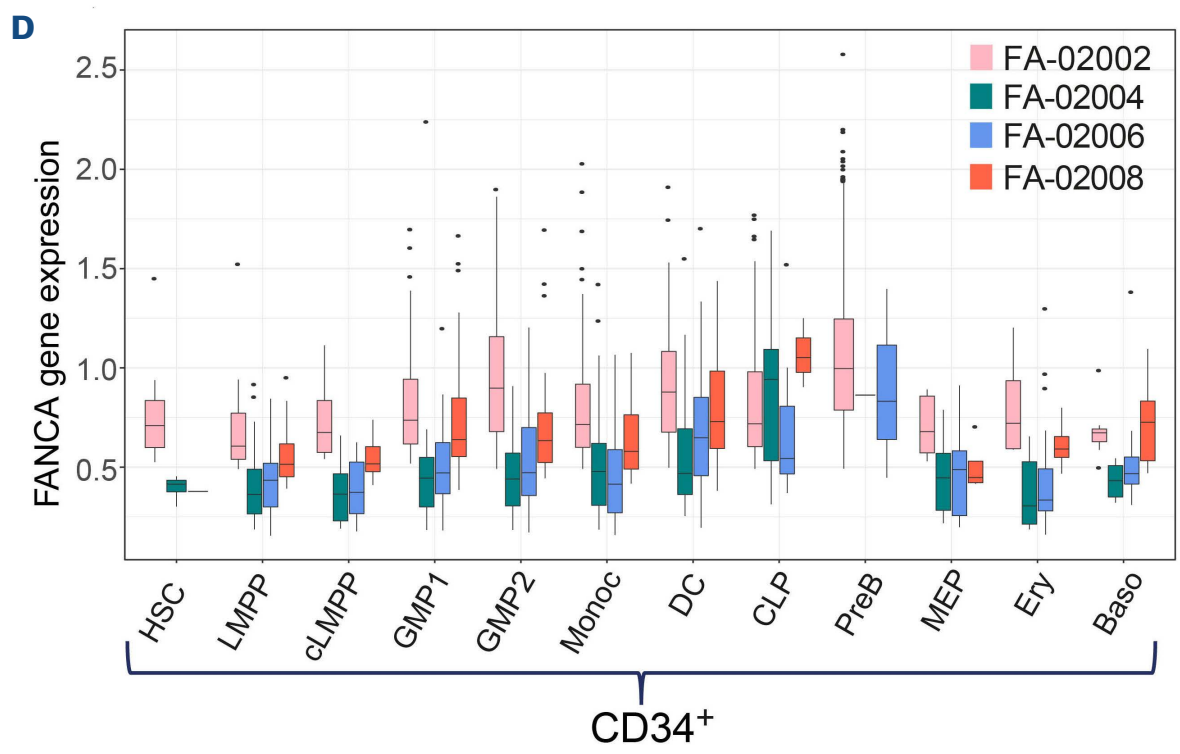
Because our analysis is based on the enrichment in FANCA$^+$ cells of gene-corrected cells, we investigated the fingerprint identified when comparing two extreme groups of cells, aiming to characterize the difference between "highly enriched gene-therapy cells (G1) *versus* non-gene-therapy cells (G2)". The first group, G1, is enriched with gene-corrected cells by including cells with at least one read mapping to: (i) FANCA; (ii) poly-A sequence of the viral vector; and (iii) PGK1. The second group, G2, is enriched with the opposite: no reads mapping to any of the previous targets. When comparing those two reduced groups of cells per sample, the differences identified by the fingerprint were also observed (*Online Supplementary Figure S2*).

Taken together, data obtained in these analyses indicate that the ectopic expression of *FANCA* induced long-term reproducible changes in the gene expression program of HSPC from FA-A patients.









Continued on following page.

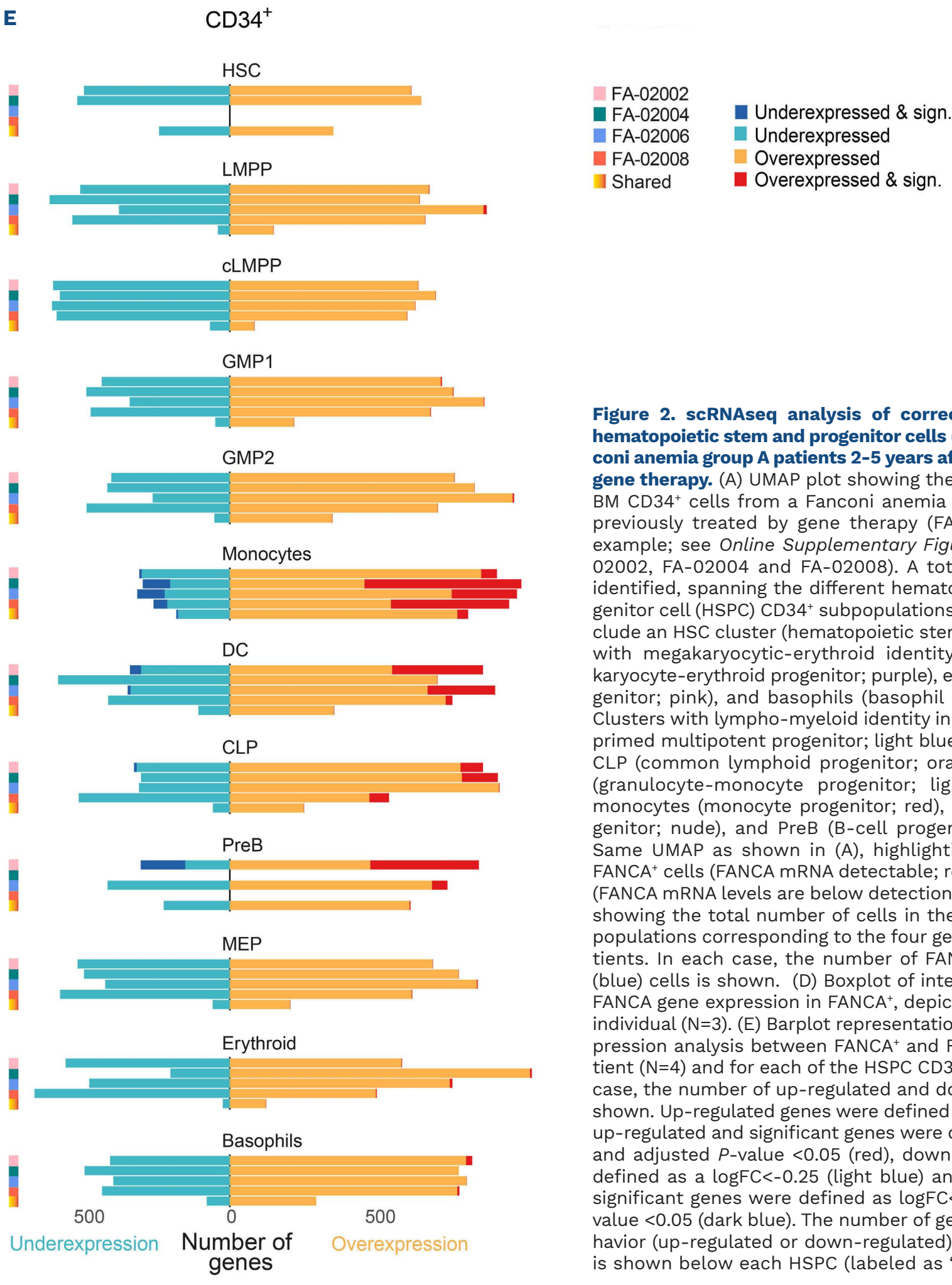
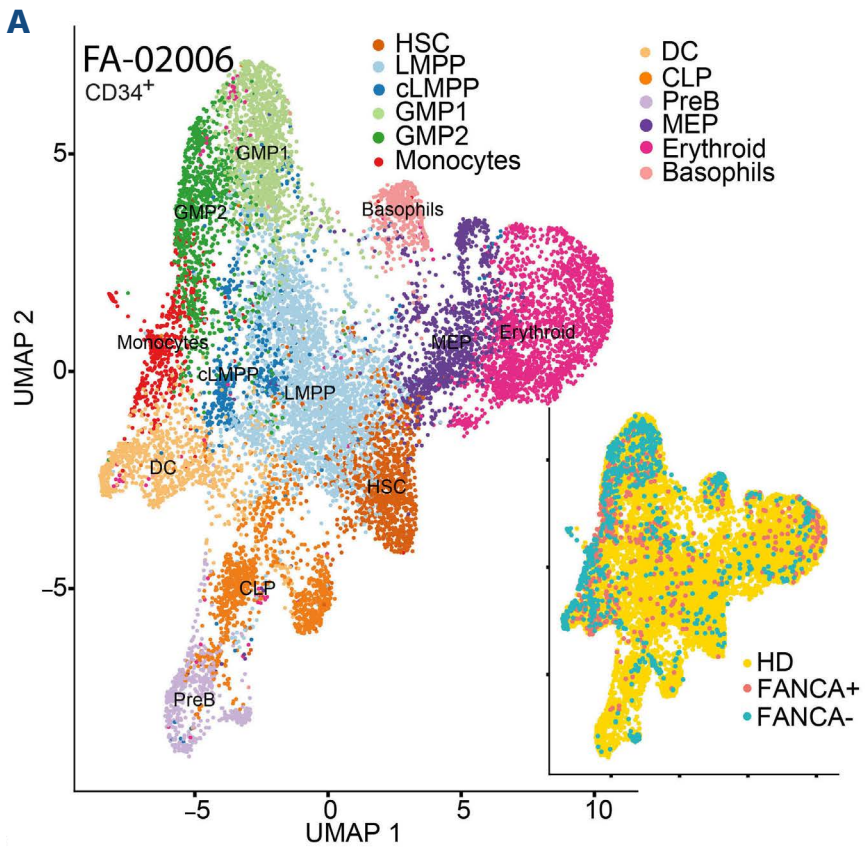
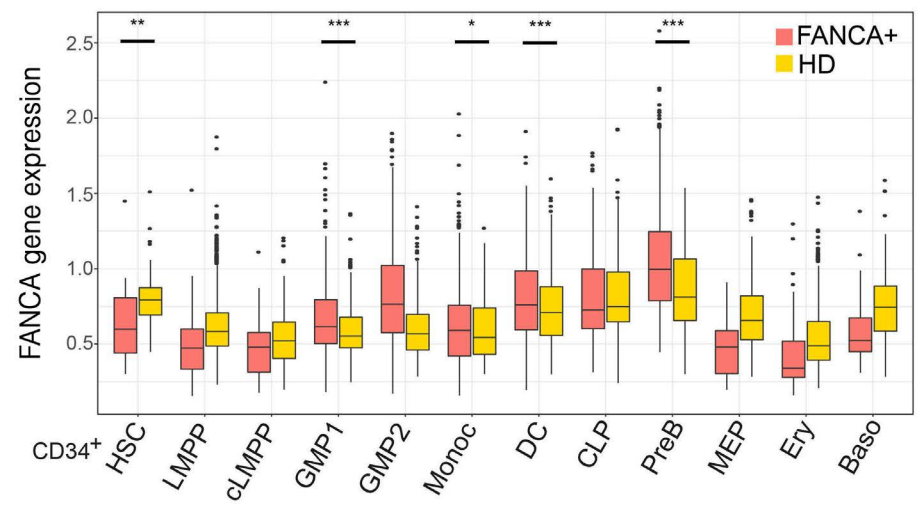


Figure 2. scRNAseq analysis of corrected and uncorrected hematopoietic stem and progenitor cells (CD34⁺ cells) from Fanconi anemia group A patients 2-5 years after lentiviral-mediated gene therapy. (A) UMAP plot showing the clustering analysis for BM CD34⁺ cells from a Fanconi anemia group A (FA-A) patient previously treated by gene therapy (FA-02006 patient as an example; see *Online Supplementary Figure S1* for patients FA-02002, FA-02004 and FA-02008). A total of 12 clusters were identified, spanning the different hematopoietic stem and progenitor cell (HSPC) CD34⁺ subpopulations. Identified clusters include an HSC cluster (hematopoietic stem cell; brown). Clusters with megakaryocytic-erythroid identity include MEP (megakaryocyte-erythroid progenitor; purple), erythroid (erythroid progenitor; pink), and basophils (basophil progenitor; light pink). Clusters with lympho-myeloid identity include LMPP (lymphoid-primed multipotent progenitor; light blue), Cycling-LMPP (blue), CLP (common lymphoid progenitor; orange), GMP1 and GMP2 (granulocyte-monocyte progenitor; light green and green), monocytes (monocyte progenitor; red), DC (dendritic cell progenitor; nude), and PreB (B-cell progenitor; light purple). (B) Same UMAP as shown in (A), highlighting the distribution of FANCA⁺ cells (FANCA mRNA detectable; red) *versus* FANCA⁻ cells (FANCA mRNA levels are below detection limit; blue). (C) Barplot showing the total number of cells in the different HSPC CD34⁺ populations corresponding to the four gene therapy-treated patients. In each case, the number of FANCA⁺ (red) and FANCA⁻ (blue) cells is shown. (D) Boxplot of integrated and normalized FANCA gene expression in FANCA⁺, depicted by cell type and FA individual (N=3). (E) Barplot representation of the differential expression analysis between FANCA⁺ and FANCA⁻ for each FA patient (N=4) and for each of the HSPC CD34⁺ populations. In each case, the number of up-regulated and down-regulated genes is shown. Up-regulated genes were defined as logFC>0.25 (orange), up-regulated and significant genes were defined as a logFC>0.25 and adjusted *P*-value <0.05 (red), down-regulated genes were defined as a logFC<-0.25 (light blue) and down-regulated and significant genes were defined as logFC<-0.25 and adjusted *P*-value <0.05 (dark blue). The number of genes with the same behavior (up-regulated or down-regulated) in the four individuals is shown below each HSPC (labeled as “shared”).

Gene therapy reprograms the transcriptional signature of Fanconi anemia hematopoietic stem and progenitor cells towards the transcriptional profile characteristic of healthy hematopoietic stem and progenitor cells

Next, we investigated whether changes in the transcriptional program of gene-corrected FA-A HSPC resembled profiles characteristic of healthy donor (HD) HSPC. To this end, scRNAseq data from GT-treated FA CD34⁺ cells was compared with those obtained from HD CD34⁺ cells. The ectopic expression of *FANCA* (FANCA⁺ cells) was detected in the different CD34⁺ cell clusters (Figure 3A and *Online Supplementary Figure S3*). In addition, *FANCA* expression levels in the





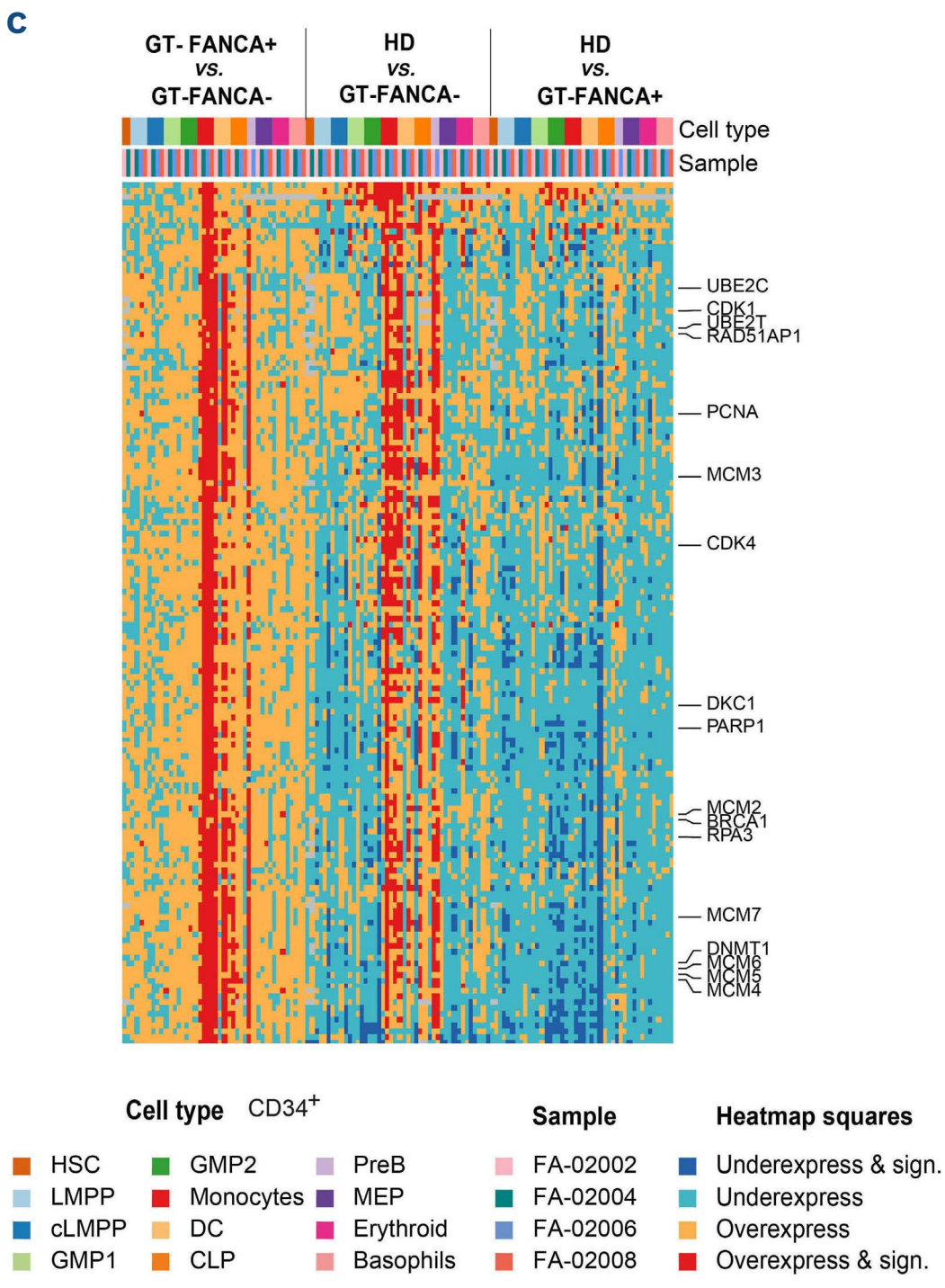


Figure 3. Comparisons of the gene expression signature between corrected and uncorrected hematopoietic stem and progenitor cells co-existing in gene therapy treated Fanconi anemia patients. (A) Left, large panel: UMAP plot showing the clustering analysis of CD34⁺ bone marrow (BM) cells after integration of data from a gene therapy-treated Fanconi anemia group A (FA-A) patient and a healthy donor (HD). (FA-02006 is included as a representative example; see *Online Supplementary Figure S3* for the other individuals). Right, small panel: the same UMAP as shown in left panel but highlighting the distribution of HD hematopoietic stem and progenitor cells (HSPC) (yellow), FANCA⁺ HSPC (red) and FANCA⁻ HSPC (blue). Cluster identification as in Figure 1. (B) Boxplot representation of normalized single-cell FANCA expression of FANCA⁺ cells (red) and HD cells (yellow) by HSPC cluster. For each cell type, differences in the expression levels between the ectopic expression of FANCA from corrected FA CD34⁺ cells and expression levels corresponding to HD CD34⁺ cells are shown. *Adjusted *P*<0.05; **adjusted *P*<0.01; ***adjusted *P*<0.001. (C) Results associated to three differential expression contrasts: FANCA⁺ *versus* FANCA⁻ HSPC from GT-treated FA patients; HD HSPC *versus* FANCA⁻ HSPC from GT-treated patients; and HD HSPC *versus* FANCA⁺ HSPC from GT-treated patients. Second row shows the twelve different CD34⁺ cell types; third row shows the sample identification from each of the four GT-treated patients. Up-regulated genes (logFC>0) are shown in orange; those with significant upregulation in red (logFC>0.25 and adjusted *P*<0.05). Down-regulated genes (logFC<0) are shown in light blue and those with significant downregulation in dark blue (logFC<-0.25 and adjusted *P*<0.05). Unsupervised hierarchical clustering using Pearson distance and average linkage method was applied for gene classification. Genes included in the heatmap are those that for at least one cell type are identified as differentially expressed (abs(logFC)>0.25 and adjusted *P*<0.05) in "at least three patients", and "showing the same direction of the change for the three patients", when considering the contrast FANCA⁺ *versus* FANCA⁻ (N=152; entire list available in *Online Supplementary Table S2*). FANCA was excluded from the list.

different HSPC subpopulations were highly heterogeneous, not only in the case of GT-treated FA patients, but also in HD (Figure 3B). Despite this heterogeneity, in 8 of the 12 HSPC subpopulation levels of FANCA mRNA were significantly higher in HD HSPC compared with the corresponding values observed in FANCA$^+$ HSPC from GT-treated FA patients. This observation is consistent with the average insertion of one proviral *FANCA* copy per corrected cell after GT, in comparison with the two copies of WT *FANCA* per HD cell, and also with the moderate activity of the phosphoglycerate kinase (PGK) promoter that had been selected for the therapeutic vector because of its favorable safety profile.

To investigate if GT reprogrammed the transcriptional signature of FA HSPC towards one corresponding to HD HSPC, additional gene expression analyses were conducted in HD and FA HSPC, focusing on the 152 genes that showed significant expression changes between FANCA$^+$ and FANCA$^-$ HSPC in GT-treated patients (Figure 2E and *Online Supplementary Table S2*). In these studies, three different comparisons were performed in each of the twelve HSPC subpopulations: FANCA$^+$ *versus* FANCA$^-$ HSPC from GT-treated FA patients (GT-FA HSPC); HD HSPC *versus* FANCA$^-$ GT-FA HSPC; and finally, HD HSPC *versus* FANCA$^+$ GT-FA HSPC (Figure 3C). Significant differences (dark red or blue spots in Figure 3C) were most evident in HSPC subpopulations with a higher representation (i.e., monocyte CD34$^+$ cells; P<0.001) (Figure 2).

Regarding the comparison of GT-FANCA$^+$ *versus* GT-FANCA$^-$ HSPC, several differentially expressed genes with relevance in FA were identified (Figure 3C). Interestingly, genes involved in functions such as DNA repair or cell cycle were up-regulated in GT-FANCA$^+$ *versus* GT-FANCA$^-$ cells. The gene expression pattern of HSPC from HD was compared with that corresponding to uncorrected HSPC from GT-treated patients (HD *vs.* GT-FANCA$^-$) (Figure 3C). Strikingly, most of the transcriptional changes observed between these populations resembled changes seen between corrected and uncorrected FA HSPC (Figure 3C; see *Online Supplementary Table S3* for detailed analyses per cell type using two complementary statistical analyses). Finally, in contrast to the above-mentioned observations, the comparison of the transcriptional program of HD HSPC *versus* FANCA$^+$ HSPC from GT-treated FA patients (Figure 3C) showed limited changes, most of which were not significant and not related to differences noted when HD or corrected FA HSPC were compared with uncorrected FA HSPC. Overall, these studies demonstrate that lentiviral-mediated GT reverts the gene expression program of FA HSPC, which then acquire an expression pattern that resembles the signature characteristic of healthy HSPC.

Lentiviral-mediated gene therapy reverts molecular pathways characteristic of Fanconi anemia hematopoietic stem and progenitor cells

Having observed that the ectopic expression of *FANCA* in FA HSPC reverts the transcriptional signature of these cells to resemble a healthy HSPC signature, we next performed a gene-set enrichment analysis to determine changes in relevant pathways associated with FA. These include pathways related to DNA damage response and repair, cell cycle checkpoint, cell aging, and telomere maintenance (see Methods). An up-regulated expression of several of these pathways was observed when FANCA$^+$ and FANCA$^-$ HSPC present in each of the GT-treated patients were compared (Figure 4 and *Online Supplementary Table S4*). Moreover, an almost identical upregulation of these pathways was observed when HSPC from HD were compared with FANCA$^-$ HSPC from GT-treated patients (Figure 4).

A deeper comparative expression analysis of genes involved in cell cycle control was then performed between FANCA$^+$ and FANCA$^-$ HSPC from GT-treated patients (Figure 5A). Similar comparisons were also made between HD HSPC and FANCA$^-$ HSPC from GT-treated patients (Figure 5A). As shown, the ectopic expression of FANCA was associated with the downregulation of *TGF*-β in every patient, and also of *p21* (*CDKNA1*) in 2 of the 4 GT-treated FA patients. On the other hand, a number of cyclins and mini-chromosome maintenance (MCM) genes were up-regulated in corrected *versus* uncorrected FA HSPC (FANCA$^+$ *vs.* FANCA$^-$ HSPC). Notably, when similar comparisons were performed between HD HSPC and FANCA$^-$ HSPC from GT-treated FA patients, almost identical gene expression changes were observed (Figure 5A), indicating that the behavior of cell cycle and DNA checkpoint pathways in corrected FA-A HSPC resembled the pathways characteristic of healthy HSPC.

Since previous studies have shown that p21 participates in the transcriptional repression of different genes of the FA/BRCA pathway,[5-8] changes in the expression of FA/BRCA genes were also comparatively investigated in FANCA$^+$ and FANCA$^-$ HSPC from GT-treated patients. In addition to FANCA, several other genes participating in the FA/BRCA pathway, including *FANCB, FANCI, FANCD2, FANCG, UB2T (FANCT), BRCA2, PALB2, BRCA1* and *BRIP1 (FANCJ)* were up-regulated in GT-FANCA$^+$ *versus* GT-FANCA$^-$ HSPC (Figure 5B). Again, many of these genes were also up-regulated when HD HSPC were compared with uncorrected GT-FANCA$^-$ HSPC.

This set of studies thus demonstrates that GT reverts physiologically-relevant molecular pathways that are severely affected in HSPC from FA patients.

Functional implications of the restored transcriptional program of corrected Fanconi anemia hematopoietic stem and progenitor cells

In a final set of experiments, we investigated the functional implications associated with the restored transcriptional program of gene-corrected FA HSPC. First, we investigated

if the restored DNA repair pathways had a direct implication in the correction of the hypersensitivity of FA-HSPC to inter-strand crosslinking agents such as mitomycin C (MMC). While colony-forming cells (CFC) from the four patients were highly sensitive to MMC prior to the infusion of corrected HSPC (no colonies were generated when cells were exposed to 10 nM MMC), survival of these cells to MMC was markedly increased 2-5 years after GT (22.3-67.3% of CFC survived in 10 nM MMC) (Figure 6A). Consistent with these studies, the percentage of T cells with DEB-induced aberrant chromosomes was also markedly decreased after GT of FA-A patients (Figure 6B), which in two patients reached

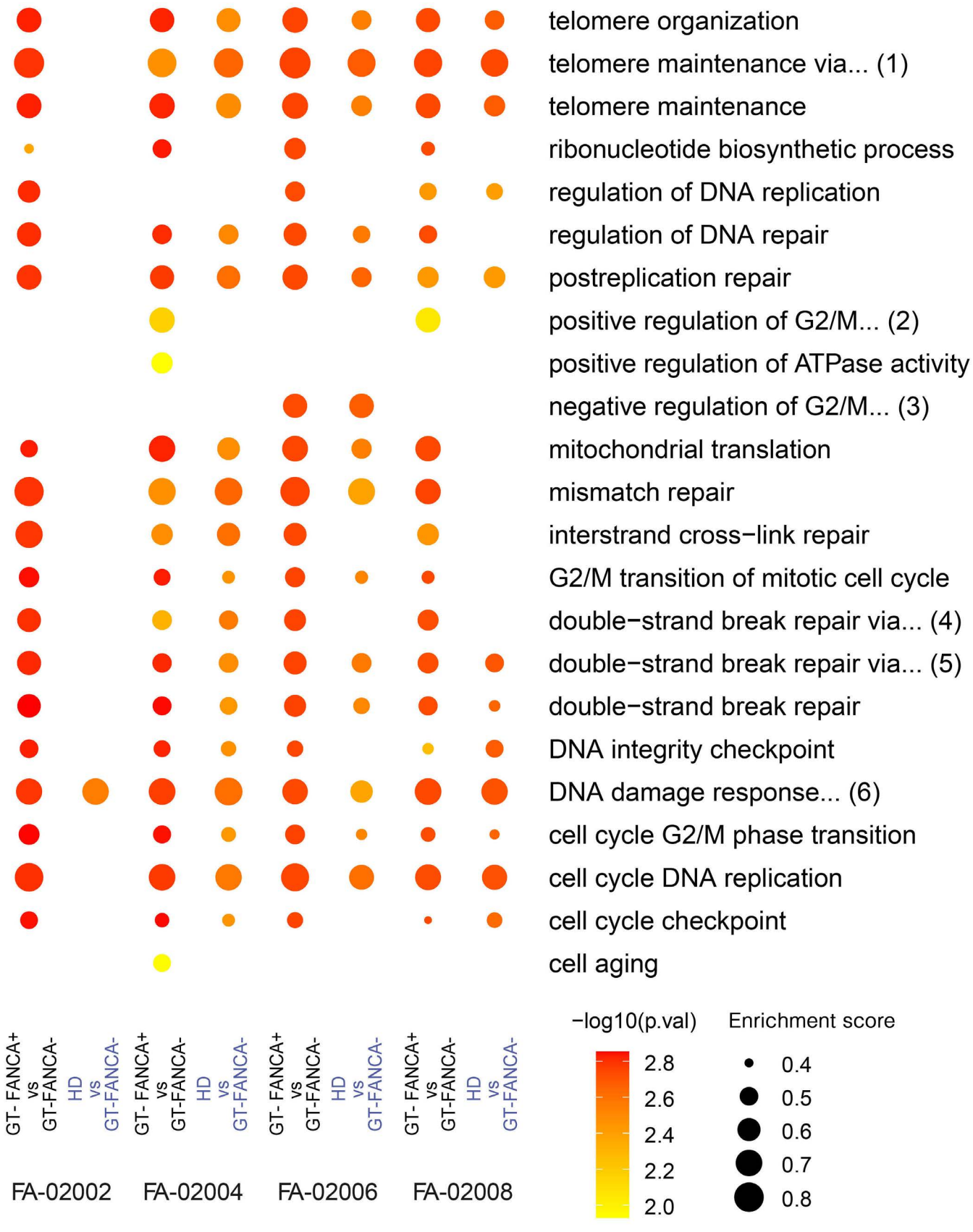


Figure 4. Enrichment score of restored gene expression pathways associated to the ectopic expression of FANCA in hematopoietic stem and progenitor cells from gene therapy-treated Fanconi anemia patients. Dotplot depicts the outcome of the Gene Set Enrichment Analysis for a selected set of pathways. For each patient, paired comparisons between FANCA^{+} *versus* FANCA^{-} hematopoietic stem and progenitor cells (HSPC) from gene therapy (GT)-treated Fanconi anemia (FA) patients and healthy donor (HD) HSPC *versus* FANCA^{-} HSPC are shown. The dotplot depicts the statistical significance (color) and enrichment score (size). The complete name of the pathways with numbers are: (i) telomere maintenance via semi-conservative replication, (ii) positive regulation of G2/M transition of mitotic cell cycle, (iii) negative regulation of G2/M transition of mitotic cell cycle, (iv) double-strand break repair via nonhomologous end joining, (v) double-strand break repair via homologous recombination, and (vi) DNA damage response, detection of DNA damage.

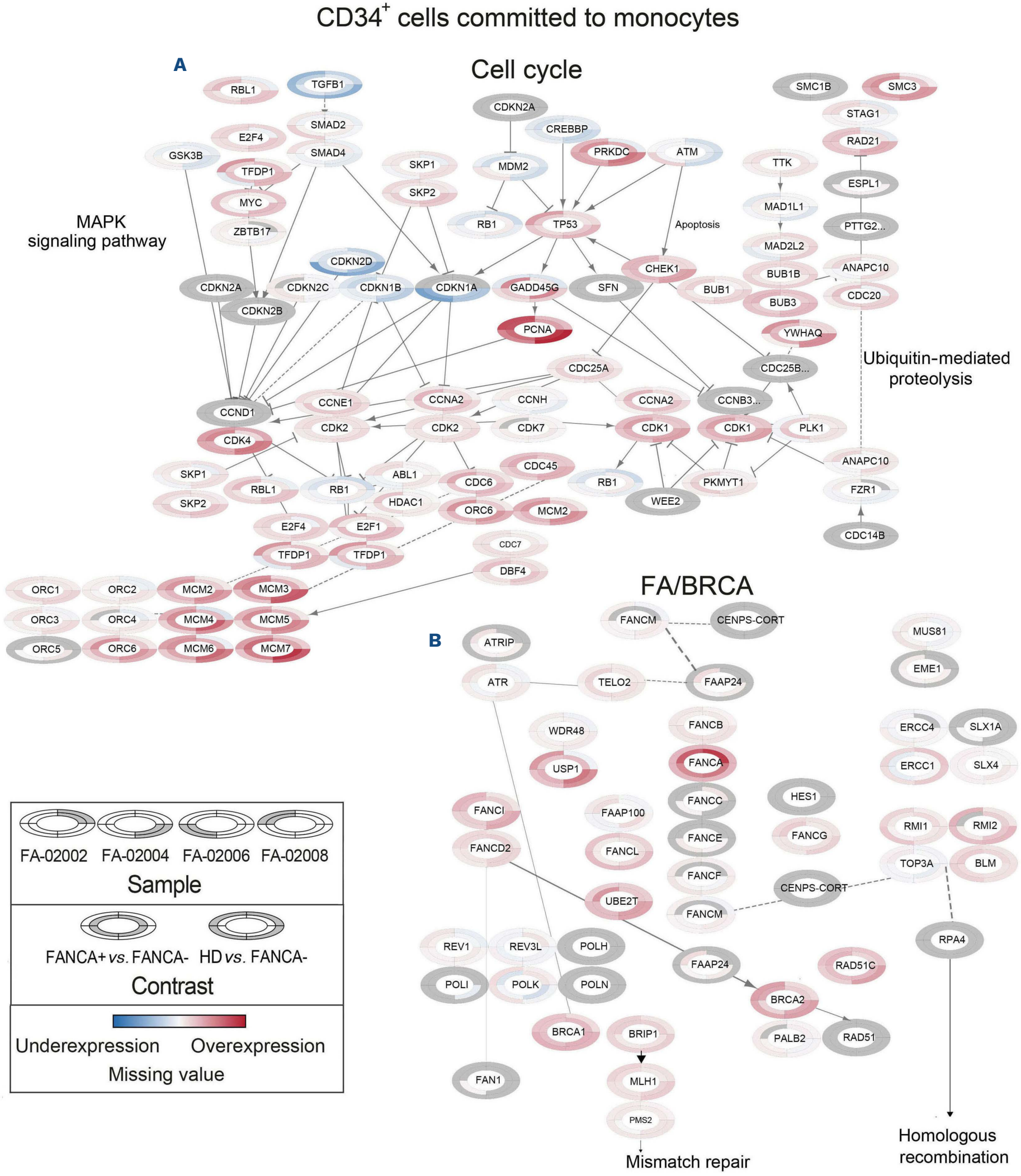


Figure 5. Restored gene expression of key pathways of CD34⁺ cells committed to the monocyte lineage in corrected hematopoietic stem and progenitor cells from gene therapy-treated Fanconi anemia patients. (A) Up-regulated and down-regulated genes implicated in cell cycle control. The figure represents the logFC associated to two contrasts (see Contrast box): FANCA⁺ *versus* FANCA⁻ hematopoietic stem and progenitor cells (HSPC) from gene therapy (GT)-treated Fanconi anemia (FA) patients (internal crowns), and healthy donor (HD) HSPC *versus* FANCA⁻ HSPC from GT-treated patients (external crowns). Each external and internal crown is divided in four parts (see Sample box), each of them representing one FA patient. Up-regulated genes are shown in red and down-regulated genes in blue. (B) Same representation as in (A) showing changes in the expression of genes participating in the FA/BRCA pathway.

levels comparable to those found in HD. Overall, these studies confirm the functional implications associated with the restoration of the DNA repair pathways observed in the scRNAseq analyses (see Figure 4).

Finally, since scRNAseq studies showed upregulation of pathways associated with the telomere elongation and stabilization (Figure 4), including *RPA3, FEN1, PARP1* and *PRKDC* (*Online Supplementary Figure S4*), we studied changes in the telomere length of PB cells from the four FA patients included in this study (Figure 6C). In particular, we evaluated changes that took place from day 15 post infusion (when the proportion of corrected cells was still undetectable) up to 5 years post infusion. As a negative control, changes in the telomere length of PB cells from three FA-A patients with no significant engraftment of corrected cells (VCN<0.008; control FA group) were investigated at comparable time points. While a clear reduction in the telomere length was observed in PB cells from the negative control group, in 2 of the 4 GT-treated patients (patients 02004 and 02006) an elongation trend was observed in PB cells at 4 years post infusion. Interestingly, the comparison of the telomere length of PB cells with HD of the same age indicated that telomeres from GT-treated patients were still below corresponding HD values. These analyses suggest that lentiviral-mediated gene therapy may be associated with the reactivation of telomere maintenance pathways, and thus with the improved preservation of telomeres from corrected hematopoietic cells.

Overall, the results obtained in this study demonstrate for the first time that GT, and in particular the lentiviral-mediated GT in FA patients, reverts the transcriptional program of diseased HSPC, which then display gene expression signatures, molecular pathways and cellular phenotypes that resemble the physiological status of healthy HSPC.

Discussion

Gene therapy has emerged as a safe and efficient therapeutic option for diverse monogenic disorders affecting the hematopoietic system.[1] In a previous study, we showed for

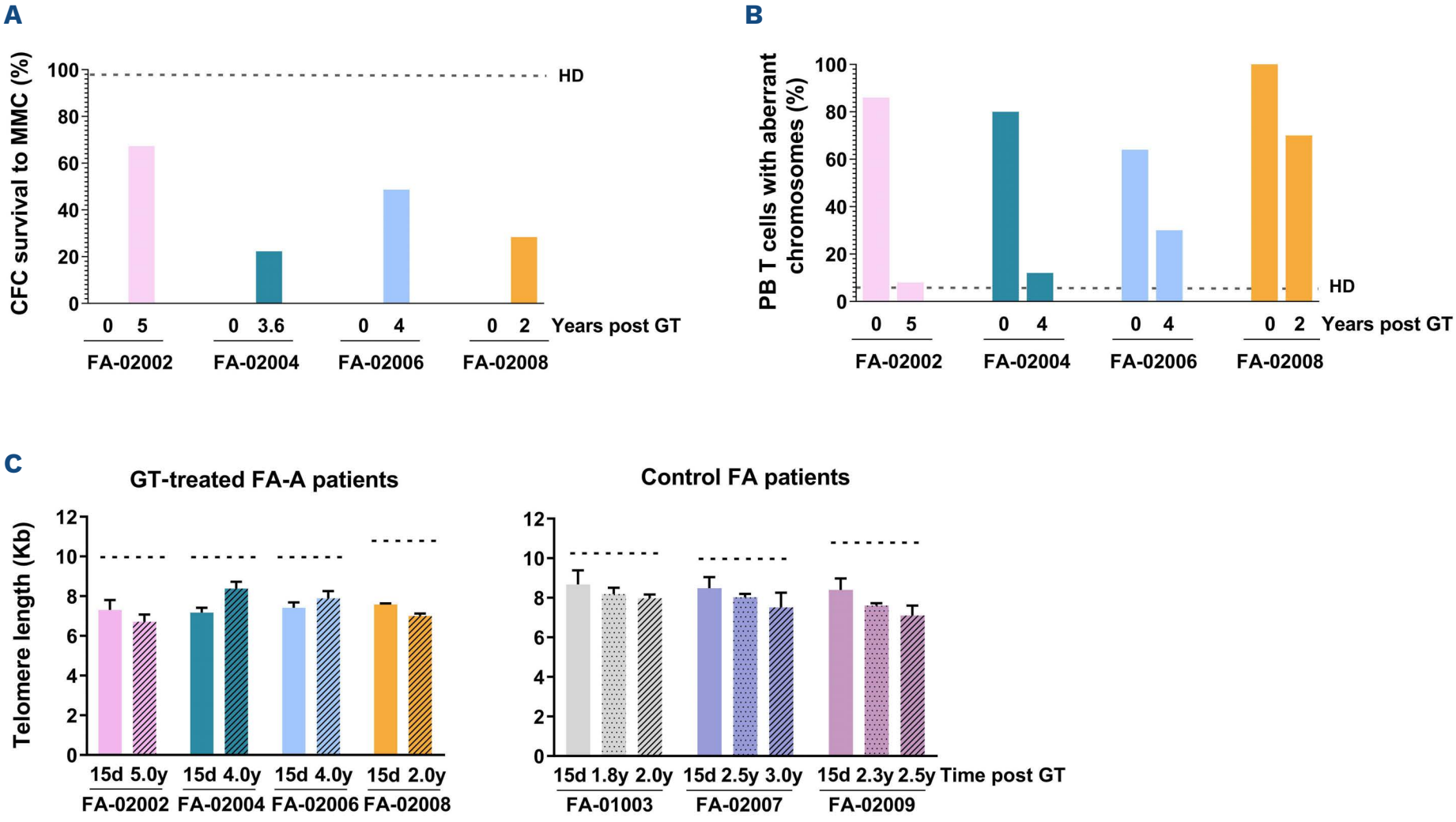


Figure 6. Functional implications associated to the restoration of DNA repair and telomere biology pathways. (A) Survival to mitomycin C (MMC; 10 nM) of bone marrow (BM) colony forming cells (CFC) from Fanconi anemia group A complementation group (FA-A) patients shown in Figures 1-5 prior and after infusion of gene-corrected cells. (B) Percentage of peripheral blood (PB) T cells with chromosomal aberrations induced by diepoxibutane (DEB), prior to and after gene therapy. (C) Analysis of the telomere length of PB cells from FA-A patients shown in Figures 1-5 at 15th day post infusion, when corrected cells were still undetectable, to the 2nd-5th year post infusion of gene-corrected cells. As a negative control, three FA patients with no significant engraftment of gene-corrected cells are included. Dashed lines correspond to telomere lengths from healthy donor (HD) age-matched PB cells. Time points of the MMC, DEB and telomere analyses are the same or the closest ones corresponding to the scRNAseq analyses shown in Figures 1-5. Percentages of survival in (A) are deduced from colony counts in cultures grown in the absence of MMC. Dashed lines (A) and (B) correspond to mean values determined in samples from HD. GT: gene therapy; d: days; y: years.

the first time preliminary clinical evidence of safety and efficacy of GT in a more complex genetic disease, such as FA.[2] In that clinical study, we demonstrated that the ectopic expression of FANCA reproducibly conferred engraftment and proliferative advantage of corrected HSC in non-conditioned FA-A patients. Nonetheless, the question of whether corrected FA HSPC acquire the transcriptional profile characteristic of healthy HSPC was not addressed in our previous study, nor in any other GT trial of a monogenic disease.

By leveraging scRNAseq profiling, we have extended the conclusions from our previous study towards the evaluation of changes in the transcriptional program of corrected *versus* uncorrected HSPC that co-exist in the BM of the same patient. These analyses, and also gene expression comparisons performed with respect to HD HSPC, allowed us to demonstrate for the first time that GT not only corrects the expression of the mutated gene (in this case, *FANCA*), but also reprograms the molecular circuits of diseased FA-A HSPC, which then acquire a normalized gene expression pattern characteristic of healthy HSPC.

Importantly, the restored expression of *FANCA* decreased the expression of two genes with particular implications in FA: *TGF*-β and *p21*. Increased mRNA levels of these two genes have been observed in HSPC from FA patients and murine FA models, and are believed to contribute to the BMF characteristic of the disease.[9,10] Notably, previous studies have shown that p21 down-regulates *USP1* upon exposure to DNA damage, disrupting FANCD2/L mono-ubiquitination and nuclear foci formation.[8] Additional studies have also shown that several genes involved in the telomere biology and in the FA/BRCA pathway are down-regulated via the p21/E2F4 pathway.[5,7] Although it might be expected that p53 and Myc, which have been shown to be up-regulated in FA patients,[9,11] should be also down-regulated in corrected FA HSPC, our data do not support this hypothesis. These unexpected observations could result either from the enhanced expression of cell cycle genes in corrected FA HSPC, with post-transcriptional regulatory mechanisms involved in p53 expression, or also from the moderate BMF status of the FA patients enrolled in the FANCOLEN I trial.[2] Our study also shows that several defective pathways implicated in the FA cellular phenotype were significantly up-regulated as a result of the ectopic expression of *FANCA* in FA-A HSPC. Collectively, the restoration of DNA repair-related pathways has enormous implications in FA GT since this accounts for the correction of the hypersensitivity of BM progenitors and PB T cells to genotoxic drugs, such as MMC and DEB.

Also of note was the observation of the increased expression of mini-chromosome maintenance (MCM) helicase genes in corrected FA-A HSPC. Previous studies have shown that decreased levels of MCM proteins are associated with HSC hypersensitivity to replication stressors,[12] and mediate a relationship between FA and accelerated aging.[13] Strikingly, our study shows for the first time that corrected FA-A HSPC up-regulates the expression of MCM, emulating the MCM expression observed in HD HSPC, and suggesting that genetic correction should reduce the replication stress and contribute to the rejuvenation of the HSC from FA patients. Importantly, our study also shows that GT up-regulates several genes associated with telomere biology, and reveals for the first time the maintenance or even elongation of the telomere length in PB cells from FA patients after 2-5 years post GT. This observation contrasts with the progressive telomere attrition previously characterized in FA patients,[14,15] and also with the negative FA control group included in our study. Although our study suggests that GT may stabilize the telomeres from gene-corrected hematopoietic cells from FA patients, longer follow-up studies should be performed in these patients to confirm the implications of GT to prevent the progressive telomere attrition characteristic of the disease, or even to extend the telomere length of corrected hematopoietic cells to levels observed in HD cells.

Our gene expression analyses in corrected and uncorrected HSPC from patients with a HSC disease, such as FA, has obvious limitations due to the low number of cells that can be analyzed. In addition, due to the limited number of individuals in whom it was possible to perform these studies, it is currently not feasible to conduct correlation studies between the transcriptomic insights and the clinical co-variates.

Taken together, in this study we show for the first time that GT reverts the transcriptional program and defective molecular pathways in corrected FA HSPC. This observation has a particular impact in FA, and possibly in other HSC diseases characterized by DNA repair defects, since here we demonstrate that GT restores the molecular pathways of FA HSPC towards a signature characteristic of healthy HSPC.

Disclosures

The Hematopoietic Innovative Therapies Division at CIEMAT receives funding from and has licensed the PGK-FANCA-WPRE lentiviral vector to Rocket Pharmaceuticals. JAB and JSe are consultants for Rocket Pharmaceuticals. EN and JDS are employees of Rocket Pharmaceuticals. PR, SN, JS and JAB are inventors on patents filed by CIEMAT, CIBERER and Fundación Jiménez Díaz, and may be entitled to receive financial benefits from the licensing of such patents. All other authors have no conflict of interests to disclose.*

Contributions

ML, PR, AV-Z, NP, SN, DA, DM, BF-V, RMJ, MA, AU-A, RP, LS and D-GC performed the experimental studies and analyzed the data. JSe and JZ provided critical materials. ML, PR, D-GC, FP and JAB wrote the manuscript. EN and JDS reviewed the manuscript. PR, SN, D-GC, FP and JAB de-

signed the study. All authors discussed the results and contributed to the preparation of the manuscript.

Acknowledgments

The authors thank Ramón García-Escudero for careful reading of the manuscript and helpful discussions. The authors also thank A. de la Cal for co-ordinating the delivery of BM samples from patients with FA. The authors are also indebted to the patients with FA, their families, and clinicians from the Fundación Anemia de Fanconi.

Funding

This work was supported by grants from the European Commission's Seventh Framework Program (HEALTH-F5-2012-305421) to the EUROFANCOLEN Consortium (to JB and JSe); from Ministerio de Ciencia, Innovación y Universidades and Fondo Europeo de Desarrollo Regional (RTI2018-097125-B-I00 and PID2021-125077OB-C21, to PR and SN); from Gobierno de Navarra, Departamento de Desarrollo Económico y Empresarial (AGATA 0011-1411-2020-000010); from Instituto de Salud Carlos III (ISCIII) and co-financed by FEDER CIBERONC (CB16/12/00489 and CB16/12/00225); Redes de Investigación Cooperativa (TERCEL RD16/0011/0005); "Instituto de Salud Carlos III (ISCIII)/Red Espanola de Terapias Avanzadas RICORS/TERAV" (RD21/0017/0027), supported by European Union-NextGenerationEU and from Consejería de Educación, Juventud y Deporte de la Comunidad de Madrid (AvanCell Project; B2017/BMD3692); from Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III, Spain, grant n. PI20/0335 and co-funded by European Regional Development (FEDER) funds. MA was supported by a PhD Fellowship from the Ministerio de Ciencia, Innovación y Universidades (FPU18/05488). CIBERER and CIBERONC are initiatives of the Instituto de Salud Carlos III and Fondo Europeo de Desarrollo Regional.

Data-sharing statement

Data supporting the findings of this study are available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180536

References

1. Ferrari G, Thrasher AJ, Aiuti A. Gene therapy using haematopoietic stem and progenitor cells. Nat Rev Genet. 2021;22(4):216-234.
2. Rio P, Navarro S, Wang W, et al. Successful engraftment of gene-corrected hematopoietic stem cells in non-conditioned patients with Fanconi anemia. Nat Med. 2019;25(9):1396-1401.
3. Gonzalez-Murillo A, Lozano ML, Alvarez L, et al. Development of lentiviral vectors with optimized transcriptional activity for the gene therapy of patients with Fanconi anemia. Hum Gene Ther. 2010;21(5):623-630.
4. Castella M, Pujol R, Callen E, et al. Chromosome fragility in patients with Fanconi anaemia: diagnostic implications and clinical impact. J Med Genet. 2011;48(4):242-250.
5. Velten L, Haas SF, Raffel S, et al. Human haematopoietic stem cell lineage commitment is a continuous process. Nat Cell Biol. 2017;19(4):271-281.
6. Zheng GX, Terry JM, Belgrader P, et al. Massively parallel digital transcriptional profiling of single cells. Nat Commun. 2017;8:14049.
7. Jaber S, Toufektchan E, Lejour V, Bardot B, Toledo F. p53 downregulates the Fanconi anaemia DNA repair pathway. Nat Commun. 2016;7:11091.
8. Rego MA, Harney JA, Mauro M, Shen M, Howlett NG. Regulation of the activation of the Fanconi anemia pathway by the p21 cyclin-dependent kinase inhibitor. Oncogene. 2012;31(3):366-375.
9. Ceccaldi R, Parmar K, Mouly E, et al. Bone marrow failure in Fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells. Cell Stem Cell. 2012;11(1):36-49.
10. Zhang H, Kozono DE, O'Connor KW, et al. TGF-β inhibition rescues hematopoietic stem cell defects and bone marrow failure in Fanconi anemia. Cell Stem Cell. 2016;18(5):668-681.
11. Rodríguez A, Zhang K, Färkkilä A, et al. MYC promotes bone marrow stem cell dysfunction in Fanconi anemia. Cell Stem Cell. 2021;28(1):33-47.
12. Flach J, Bakker ST, Mohrin M, et al. Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. Nature. 2014;512(7513):198-202.
13. Brosh RM Jr, Bellani M, Liu Y, Seidman MM. Fanconi anemia: a DNA repair disorder characterized by accelerated decline of the hematopoietic stem cell compartment and other features of aging. Ageing Res Rev. 2017;33:67-75.
14. Leteurtre F, Li X, Guardiola P, et al. Accelerated telomere shortening and telomerase activation in Fanconi's anaemia. Br J Haematol. 1999;105(4):883-893.
15. Hanson H, Mathew CG, Docherty Z, Mackie Ogilvie C. Telomere shortening in Fanconi anaemia demonstrated by a direct FISH approach. Cytogenet Cell Genet. 2001;93(3-4):203-206.