Granulocyte colony-stimulating factor produces long-term changes in gene and microRNA expression profiles in CD34+ cells from healthy donors

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ABSTRACT

Granulocyte colony-stimulating factor is the most commonly used cytokine for the mobilization of hematopoietic progenitor cells from healthy donors for allogeneic stem cell transplantation. Although the administration of this cytokine is considered safe, knowledge about its long-term effects, especially in hematopoietic progenitor cells, is limited. On this background, the aim of our study was to analyze whether or not granulocyte colony-stimulating factor induces changes in gene and microRNA expression profiles in hematopoietic progenitor cells from healthy donors, and to determine whether or not these changes persist in the long-term. For this purpose, we analyzed the whole genome expression profile and the expression of 384 microRNA in CD34+ cells isolated from peripheral blood of six healthy donors, before mobilization and at 5, 30 and 365 days after mobilization with granulocyte colony-stimulating factor. Six microRNA were differentially expressed at all time points analyzed after mobilization treatment as compared to the expression in samples obtained before exposure to the drug. In addition, 2424 genes were also differentially expressed for at least 1 year after mobilization. Of interest, 109 of these genes are targets of the differentially expressed microRNA also identified in this study. These data strongly suggest that granulocyte colony-stimulating factor modifies gene and microRNA expression profiles in hematopoietic progenitor cells from healthy donors. Remarkably, some changes are present from early time-points and persist for at least 1 year after exposure to the drug. This effect on hematopoietic progenitor cells has not been previously reported.

Introduction

The mobilization of hematopoietic progenitor cells (HPC) for hematopoietic transplantation from bone marrow to blood stream by growth factors was introduced into clinical practice in the 1980s.[1-4] Granulocyte colony-stimulating factor (G-CSF) is the most commonly used cytokine and it is administered worldwide to thousands of patients and healthy donors every year. Several studies, in which different molecular and clinical parameters were analyzed for 10 years after G-CSF administration, have been reported describing its safety profile among healthy donors. In this regard, no increase in the incidence of hematologic malignancies was detected as compared to that in the normal population.[5,6] Furthermore, the donors' early post-donation quality of life following the collection of HPC from peripheral blood is better than that following bone marrow donation.[7] At the molecular level, G-CSF induces the release of proteolytic enzymes from neutrophils into the extravascular compartment of the bone marrow. These enzymes degrade and inactivate the linkage proteins between HPC and the bone marrow stroma releasing the HPC into the peripheral blood. The SDF-1/CXCR4 axis includes some of the most important receptors involved in the interaction between HPC and stroma.[8-13] It is known that G-CSF causes a decrease in the expression of SDF-1 in the bone marrow stroma.[14] Nevertheless, there is not much information about the effects of G-CSF on the expression of other genes in HPC.

Several studies have shown that microRNA (miRNA) may also play an important role in the mobilization of HPC. Indeed, HPC have different miRNA expression profiles depending on whether they are mobilized with G-CSF or plerixafor.[15,16] miRNA are involved in different biological processes, including development, differentiation, proliferation and cell death.[17] Accordingly, changes in the expression of miRNA associated with the drug-induced mobilization of HPC might lead to changes in gene expression. In this regard, it has been described that G-CSF induces changes in both gene and miRNA expression profiles in leukocytes for up to 9 months after mobilization.[18-20] Its long-term effects on HPC do, however, still need to be analyzed.

On this background, we hypothesized that G-CSF could modify gene and miRNA expression profiles of HPC, resulting in changes that could affect these cells' biological features. The aim of this study was, therefore, to analyze, with high throughput techniques, whether G-CSF induces changes in

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gene and miRNA expression profiles in HPC from healthy donors, and to determine whether any changes in expression signatures persist in the long-term or return to the original status.

Methods

Samples

CD34+ progenitor cells from peripheral blood of six healthy donors were collected before and at 5, 30 and 365 days after the mobilization with G-CSF (mobilization regimen: 10-15 μg/kg of G-CSF daily for 5 days). All donors were included in the transplant program of the Hematology Department of the University Hospital Virgen del Rocío (Seville, Spain). The local ethics committee of the same hospital provided institutional review board-approval for this study, and informed consent was obtained from all donors in accordance with the Declaration of Helsinki.

Isolation of hematopoietic progenitor cells

Mononuclear cells were collected from all samples by density gradient centrifugation with Ficoll-Paque solution (Amersham Biosciences, Uppsala, Sweden). The CD34+ cells were isolated in an AutoMACS pro separator (Miltenyi Biotec, Bergisch Gladbach, Germany) by positive immunomagnetic selection using the CD34 MACS microbead Human Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and, after magnetic enrichment, CD34+ cells were sorted by flow cytometry. The purity of the isolated CD34+ cells was greater than 95% in all cases.

RNA extraction

Total RNA was extracted by TRIsure (Bioline, Luckenwalde, Germany) in all samples. The quality and integrity of the RNA were verified by a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

MicroRNA and gene expression

The expression profiles of 384 miRNA were analyzed in all samples using TaqMan Human MicroRNA v2.0 Arrays (Applied Biosystems, Foster City, CA. USA) which were analyzed on a 7900 HT Fast Real Time Polymerase Chain Reaction (PCR) System (Applied Biosystems, Foster City, CA, USA). The SDS 2.3 and RQ Manager 1.2 software (both Applied Biosystems, Foster City, CA, USA) were used for the analysis. Data were normalized using the average of the endogenous small-nucleolar RNU48 and the non-coding small nuclear U6, both included in the array.

The expression profile of 45,000 genes was analyzed in the same samples using the Whole Human Genome Oligo microarray kit 4x44K (Agilent Technologies, Santa Clara, CA, USA). The microarrays were scanned in a GenePix reader (Molecular Devices, Sunnyvale, CA, USA). Samples from non-mobilized CD34+ cells were used as the reference group in both types of expression analysis.

The expression of significant genes was validated by quantitative real-time PCR using Quantitec Primer Assays and the Quantitec SYBR Green Kit (both from Qiagen, Hilden, Germany) in a 7900 HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were normalized to the housekeeping gene *ACTB* and the group of samples from non-mobilized CD34+ cells was used as a control. The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Unsupervised hierarchical clustering of gene and miRNA expression data was performed using the average linkage and the Euclidean distance. To identify the genes and miRNA differentially expressed in CD34+ cells before and at the different time-points after the G-CSF administration we applied the non-parametric Mann-Whitney test. To obtain positive and negative expression values, data were transformed to a logarithmic scale. All analyses were performed using Multi-experiment Viewer 4.7.1 software. The function of the genes and miRNA of interest was determined from different databases available online (miRbase, Gene Ontology, Ingenuity Pathways Analysis).

Results

Effect of granulocyte colony-stimulating factor on microRNA expression of hematopoietic progenitor cells

miRNA expression profiles were determined in CD34+ cells from peripheral blood before and at different time-points after mobilization with G-CSF. Seventy-two out of 384 miRNA were undetectable across all samples and were excluded from further analyses. We performed unsupervised paired hierarchical clustering comparing the expression of the miRNA from non-mobilized CD34+ cells *versus* those obtained at 5, 30 or 365 days after exposure to the drug. In all paired comparisons two groups were clearly differentiated. One of them included the non-mobilized samples and the other one contained those samples obtained at the different time-points post-mobilization, thus indicating that G-CSF causes changes in miRNA expression patterns until at least 1 year after mobilization. To identify those miRNA differentially expressed between the different groups, a non-parametric Mann-Whitney test was applied. All miRNA with a P value <0.05 were considered statistically significant. Figure 1 shows paired hierarchical clusters including only differentially expressed miRNA.

At day 5 after treatment, we identified 15 differentially expressed miRNA, 12 of which were over-expressed and three under-expressed as compared to their expression in non-mobilized CD34+ cells (Figure 1A).

At day 30, we found 179 differentially expressed miRNA, 177 over-expressed and two under-expressed (Figure 1B). Out of these, nine miRNA were also over-expressed at day 5 after mobilization, whereas 168 miRNA appeared over-expressed for the first time at day 30 of the mobilization.

One year after G-CSF administration, we identified 155 differentially expressed miRNA, 130 of which were over-expressed and 25 under-expressed as compared to the expression level in the control (Figure 1C). Among all of these miRNA, 128 were also over-expressed at day 30 and maintained their expression levels 1 year after G-CSF administration, while six miRNA (miR182, miR21, miR339-3p, miR483-5p, miR500 and miR576-3p) showed over-expression since the start of the treatment and for up to 1 year later (Figure 2). These miRNA are mainly involved in processes such as cell cycle, proliferation, angiogenesis and immune response (Table 1). Overall, we found that G-CSF induced mostly over-expression of miRNA in mobilized HPC. The highest number of over-expressed miRNA was observed on day 30 after G-CSF administration and the same trend remained for at least 1 (Figure 1D).

In addition, in order to check that the miRNA expression pattern of CD34+ cells from peripheral blood does not vary

over time in normal conditions, we performed the same unsupervised hierarchical clustering comparing the miRNA expression profiles of CD34⁺ cells from peripheral blood of six control subjects, collected before and 30 days after administration of a vehicle (saline solution). As we expected there were no changes in a subject's expression pattern of miRNA over time (*Online Supplementary Figure S1*).

Effect of granulocyte colony-stimulating factor on protein-encoding gene expression of hematopoietic progenitor cells

We performed high-throughput gene expression analysis of the same samples by microarray technology. Once again, unsupervised hierarchical clustering comparing non-mobilized samples with each of those collected at the different time-points after mobilization clearly identified two clusters which included the samples obtained before and after exposure to G-CSF. Interestingly, most of the differences in treated samples remained 1 year after mobilization. In order to identify those genes differentially expressed in CD34⁺ cells before G-CSF and at the different time-points after mobilization, we applied a non-parametric Mann-Whitney test in all paired unsupervised analyses. The differences in expression with a P value <0.05 were considered statistically significant.

We identified 4136 genes differentially expressed in non-mobilized CD34⁺ cells *versus* CD34⁺ cells obtained 5 days after exposure to G-CSF (Figure 3A). Out of these, 2113 genes were up-regulated and 2023 were down-regulated as compared to non-mobilized samples.

Thirty days after G-CSF administration we found 4960 differentially expressed genes: as compared to their expression in untreated samples, 1848 were up-regulated and 3112 were down-regulated 30 days after mobilization (Figure 3B). Of all these genes, 1899 appeared differentially expressed for the first time at day 30 of the mobilization, 635 being up-regulated and 1264 being down-regulated.

Finally, upon comparing CD34⁺ cells before and 1 year after mobilization, we found 4805 differentially expressed genes: 1969 were up-regulated and 2836 down-regulated (Figure 3C). In this case 2381 genes appeared differentially expressed for the first time 1 year after G-CSF treatment:

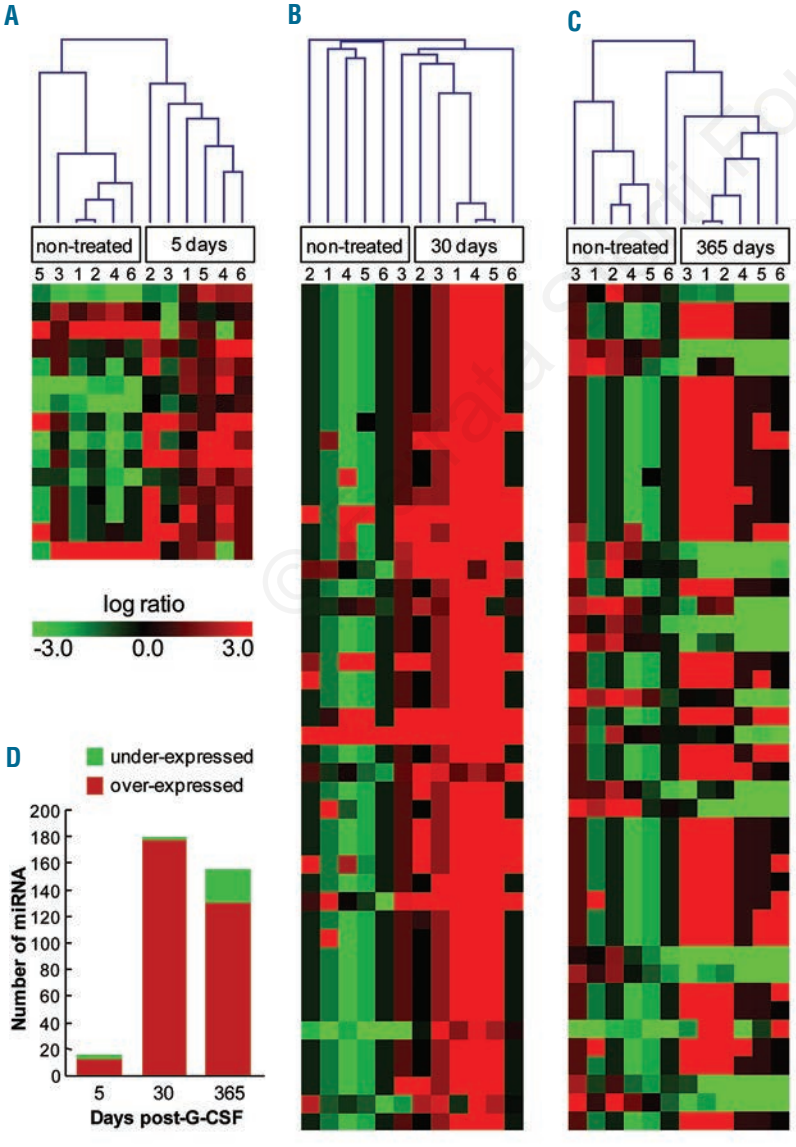

Figure 1. Unsupervised hierarchical cluster analysis of miRNA. The analysis was performed with the miRNA differentially expressed between CD34⁺ cells from non-treated samples and at 5 days (A), 30 days (B), and 1 year (C) after G-CSF administration. Each numbered column represents an individual sample and each row represents a single miRNA. Panel A contains all the miRNA included in the analysis. Panels (B) and (C) contain representative portions of the respective sets of miRNA. The red and green color code indicates miRNA expression levels in logarithmic scale. The statistical analysis was performed using the non-parametric Mann-Whitney test. (D) Number of over- and under-expressed miRNA at the different time-points after G-CSF administration relative to expression in non-treated samples.

1075 were up-regulated and 1306 were down-regulated.

Among all of the differentially expressed genes, 2424 maintained their expression levels from day 5 to 1 year after the treatment with G-CSF; 894 of these genes were up-regulated and 1530 down-regulated as compared to levels in non-mobilized CD34+ cells. In order to analyze those genes whose expression levels remained altered at all time-points analyzed, we set a cut-off value of 1.5 above or below the expression of controls. Using this cut-off we identified 617 genes, of which 232 were up-regulated and 385 were down-regulated after mobilization, as compared to levels in the control. Functional analysis of these genes using Ingenuity Pathway Analysis software (*http://www.ingenuity.com*) showed that these genes were involved in several biological processes such as cancer, gene expression, protein synthesis, cellular growth and proliferation, cell death and survival, cell cycle and hematopoiesis (Table 2).

These results showed that G-CSF alters the expression profiles of genes in mobilized HPC. As for the miRNA, the highest number of up- or down-regulated genes was observed after 30 days of treatment. Again, most of the changes persisted for at least 1 year after mobilization (Figure 3D).

Furthermore, we compared the gene expression patterns of CD34+ cells from the peripheral blood of six control subjects, collected before and 30 days after the administration of a vehicle (saline solution). As observed for miRNA, gene expression levels did not vary over time in individual normal subjects (*Online Supplementary Figure S2*).

Finally, in order to validate the high-throughput screening, we confirmed the expression of eight out of these 617 genes (*CCL3L3*, *SCIMP*, *FGF3*, *MAP4K1*, *EEF1A2*, *IRF2BP2*, *BNIP3L* and *RPS27*) by quantitative real-time PCR (Figure 4). These genes were selected because, apart from showing a strong regulation, each of them participates in a different biological process. *CCL3L3* is related to cell proliferation and immune responses, *FGF3* participates in cell growth and proliferation, *SCIMP* is involved in the regulation of antigen presentation or cell activation, *MAP4K1* plays an important role in hematopoiesis and hematologic system

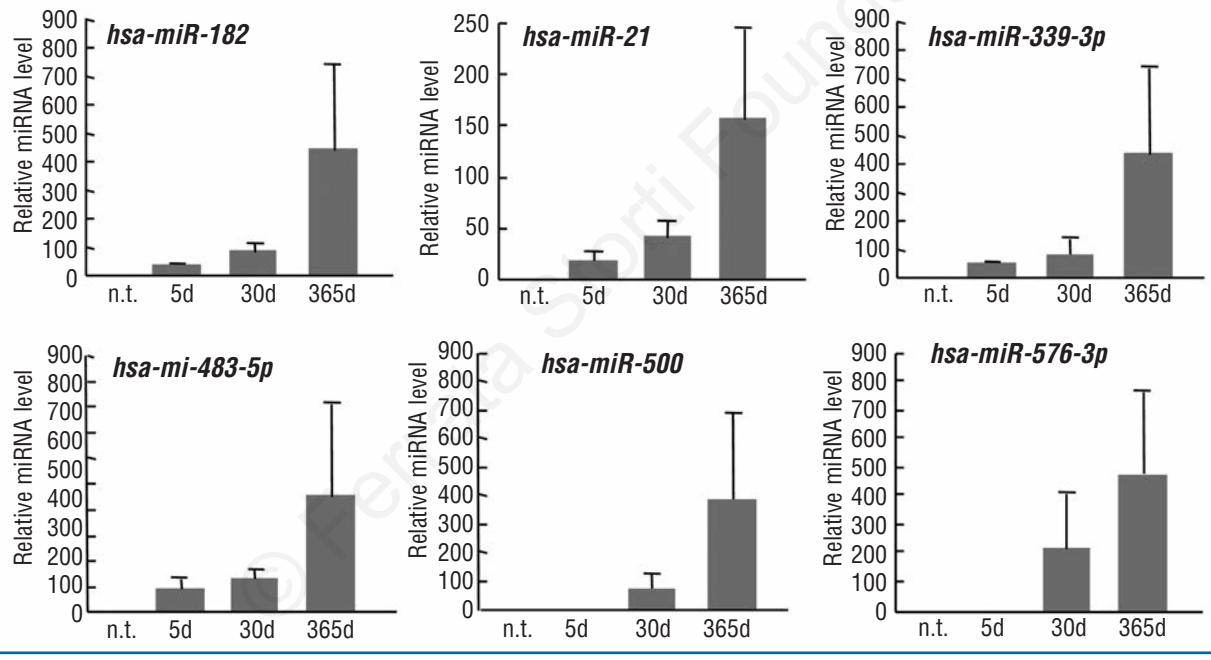

Figure 2. Expression levels of differentially expressed miRNA at all three time-points analyzed after G-CSF administration. Differences in miRNA expression at 5, 30, and 365 days (d) relative to the expression in non-treated (n.t.) samples. Statistical significance: *$P<0.05$.

Table 1. Differentially expressed miRNA after G-CSF mobilization.

miRNA	Relative expression (log ratio)				Biological process
	Untreated	5 days	30 days	365 days	
hsa-miR-182	-0.92	1.44	3.62	4.07	Cell growth, proliferation and cell cycle
hsa-miR-21	-3.84	0.74	1.68	1.62	
hsa-miR-339-3p	-2.21	1.94	3.05	3.39	Angiogenesis, apoptosis, cell cycle, proliferation, stemness and immune response
hsa-miR-483-5p	-0.92	4.50	5.42	4.75	Angiogenesis, proliferation and cell cycle
hsa-miR-500	-0.87	1.50	3.78	4.07	Immune response and inflammation
hsa-miR-576-3p	-0.92	2.67	4.55	4.07	Translation process

Expression levels of the differentially expressed miRNA at all time-points analyzed after G-CSF mobilization relative to expression in non-treated samples, and biological processes in which they are implicated. Statistical significance: $P<0.05$.

development and function, and *EEF1A2* is a translation elongation factor implicated in cell death and survival. Moreover, *CCL3L3*, *SCIMP*, *FGF3*, *MAP4K1* and *EEF1A2* showed up-regulation post-mobilization and, interestingly, they were even more over-expressed 1 year after mobilization. In addition, *IRF2BP2*, *BNIP3L* and *RPS27* were also selected because, according to the different databases available in internet (*http://www.targetscan.org* and *http://www.mirbase.org*), they are targets of the differentially expressed miRNA identified in the current study. These three genes appeared down-regulated after the treatment as compared to the controls. *IRF2BP2* is a growth factor and *BNIP3L* is a tumor suppressor that inhibits cell proliferation. Finally, *RPS27* participates in the synthesis of proteins and its down-regulation is related to some hematologic diseases.

Discussion

The use of G-CSF is considered safe, according to several studies which have not found an increased risk of hematologic diseases among donors exposed to the drug; however, there are no studies analyzing its effects on gene and

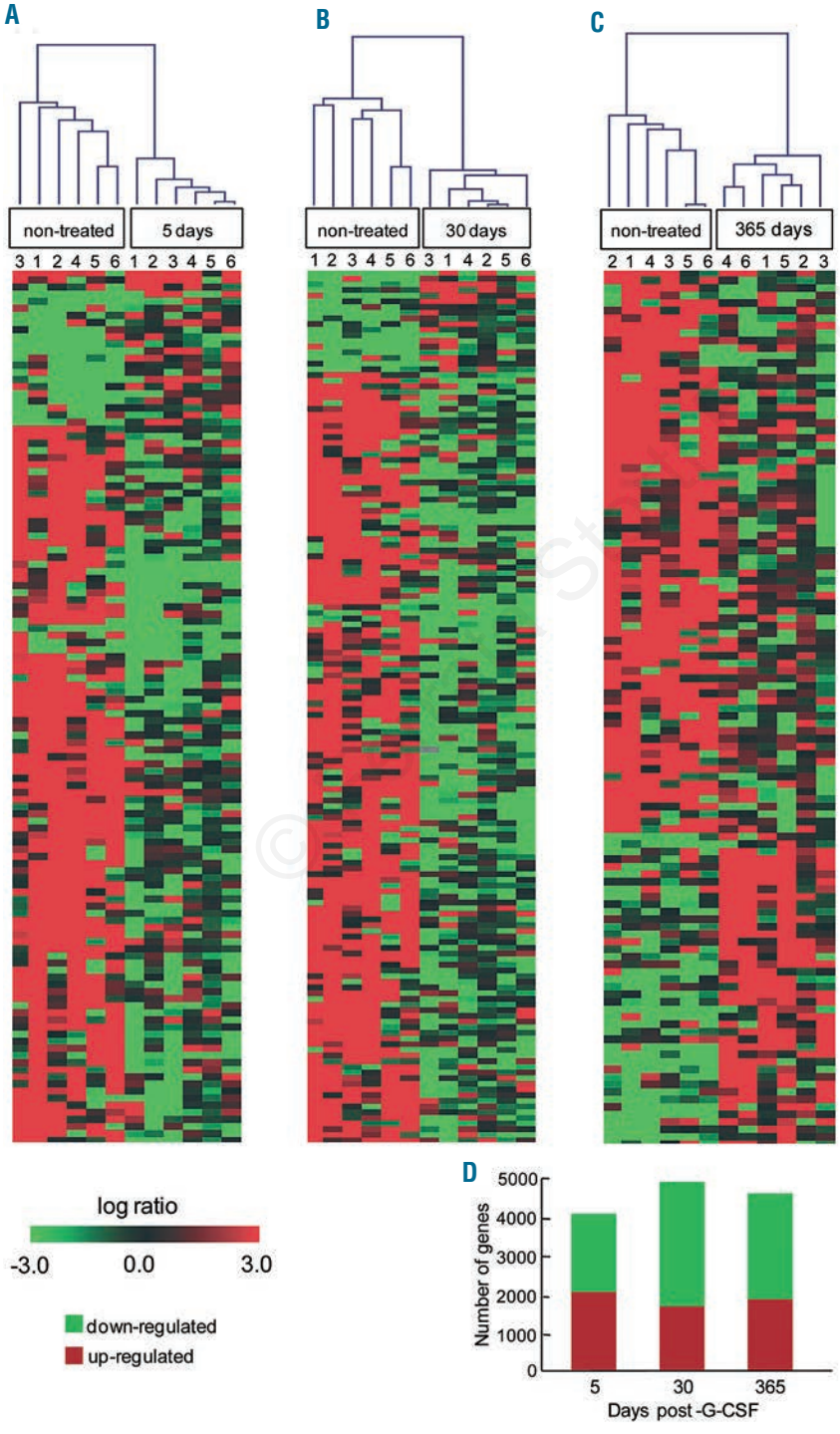

Figure 3. Unsupervised hierarchical cluster analysis of genes. The analysis was performed with the genes differentially expressed between CD34$^+$ cells from non-treated samples and at 5 days (A), 30 days (B), and 1 year (C) after G-CSF administration. Each numbered column represents an individual sample and each row represents a single gene. Panels contain representative portions of the respective sets of genes. The red and green color code indicates gene expression levels in logarithmic scale. Statistical analysis was performed using the non-parametric Mann-Whitney test. (D) Number of up- and down-regulated genes at the different times after G-CSF administration relative to non-treated samples.

Table 2. Differentially expressed genes after G-CSF mobilization.

Top function		Focus Molecules	Expression Levels	
			Down-regulated	Up-regulated
Diseases and disorders	Cancer	21	AKIRIN2, ATF3, CASP3, CAST, CSNK1E, CXCL2, EGR1, FOS, GNAS, HIF1A, KLF6, NFKBIA, NPM1, PRNP, RRM1, TXNIP	CACYBP, CD82, DUSP2, EIF2AK2, FGF3
	RNA post-transcriptional modification	31	AKAP17A, C1QBP, EXOSC7, FBL, HNRNPK, HNRNPM, INTS10, MBNL1, NPM1, PNN, RBM39, RBMS1, RPL14, RPL26, RPL7, RPS15, RPS27, RPS28, RPS6, RPS7, SNRPC, SNRPD1, SRSF2, SSB, SYNCRIP, TRA2B, WDR55, YTHDC1	APLP1, POLR2A, RNGTT, SNRNP70
	Protein synthesis	41	BCR, CASP3, EEF1B2, EIF1, EIF3D, EIF3K, EIF3L, EIF4B, EIF4H, FOS, GSK3A, HNRNPK, KLF2, NACA, NPM1, PPP1R2, PRNP, RPL13A, RPL17, RPL24, RPL30, RPL37, RPL39, RPS15A, RPS27, RPS29, RPS6, RPS7, SERINC1, SOD1, SSB, SYNCRIP, TNIP1, TNRC6B	APLP1, EEF1A2, EIF2AK2, IGFBP3, LIF, PASK, SNRNP70,WIBG
	Gene expression	109	AES, AKIRIN2, ATF3, BAG1, BCLAF1, BCR, BPTF, BTG3, C14orf166, C1QBP, CBX5, CCNH, CITED2, CSNK1E, DCP1A, DNAJB6, DUSP1, E2F3, EAPP, EGR1, EIF1, EIF3D, EIF3K, EIF3L, EIF4B, EIF4H, FOS, HIF1A, HINT1, HIPK1, HMGN1, HMGN2, HOPX, ID1ID2, IGBP1, IKZF2, ILF2, INPP5D, KLF2, KLF6, KLF9, KPNA2, MATR3, MED26, NACA, NAE1, NDNL2, NFKBIA, NPM1, POLA1, PSIP1, PTGES3, PURB, RBBP6, RBM39, RCOR1, RPL13A, RPL17, RPL24, RPL30, RPL37, RPL39, RPL6, RPS27, RPS29, S1PR1, SAP18, SATB1, SSB, STRAP, SUB1, SUPT4H1, SYNCRIP, TAF6, TCEB1, TDG, TNIP1, TRAF6, TXNIP, UBE2I, VAPA, WRN, YWHAQ, ZMIZ1	APLP1, BHLHE23, CCL3L1, CD3EAP, CD82, CHRM1, EIF2AK2, GPR183, HOXB4, HOXB7, IGFBP3, LIF, MYBL2, NFE2L1, NFIA, PHOX2A, POU3F4, PRKG1, SIK1, SP100, TEAD3, TNFRSF1A, ZNF467, ZNF496
Molecular and cellular functions	Nucleic acid metabolism	19	ATP2A2, ATP5E, ATP5F1, ATP5O,ATP6V0C, CYCS, HIF1A, IMPDH1, MAP1LC3B, MSH6, NT5C2, SLC25A5, SOD1, TXNIP, WRN	ACTC1, AK4, ASK, PPP2R4
	Cellular growth and proliferation	179	ACTB, ADAR, AES, AHNAK, AKIRIN2, AMBRA1, ANXA2, ARF1, ARL2BP, ATF3, ATP2A2, ATP5F1, BAG1, BBC3, BCLAF1, BCR, BNIP3L, BRK1, BTG3, C1QBP, CALCOCO2, CASP3, CAST, CCNH, CCT2, CD69, CDK6, CHKA, CITED2, CNP, CSNK1E, CXCL2, DNAJB6, DUSP1, E2F3, EAPP, EEF1B2, EGR1, EIF1, EIF4B, FBRS, FOS, GNAS, GNE, GNL3, H2AFY, HIF1A, HINT1, HIPK1, HK2, HLA-DPB1, HLA-DQB1, HNRNPK, HNRNPM, HOPX, HOXA9, HPGDS, ID1, ID2, IGBP1, IGF1R, IKZF2, ILF2, ILKAP, IMPDH1, INPP5D, IRF2PB2, KDM6A, KLF2, KLF6, KLF9, KPNA2, LTB, MCFD2, MLLT3, MSH6, NAA30, NACA, NAE1, NAP1L1, NDE1, NDNL2, NFKBIA, NPM1, NUP98, PNN, POLA1, PRKCSH, PRNP, PSMF1, PTGES3, PTP4A2, RANBP9, RBBP6, RGCC, RHEB, RPL26, RPS15A, RPS6, RRM1, S100A13, S1PR1, SATB1, SKP1, SLC25A5, SLC9A3R1, SMYD3, SOD1, SRSF2, STRAP, SUGT1, TAF6, TCP1, TOP1MT, TRAF6, TSPAN3, TXNDC5, TXNIP, UBC, UBE2E3, UBE2I, WRN, WTAP, YME1L1, YWHAQ, ZMIZ1, ZSCAN18	AK4, ANGPTL6, AVPR1B, AXL, CACNA1A, CACYBP, CCL3L3, CD248, CD82, CHRM1, CKLF, CLDN15, CRHR1, CX3CL1, DBF4B, DPT, DUSP2, EIF2AK2, FGF3, GAPT, GAS2L1, GNG4, GPR183, HOXB4, HOXB7, IGFBP3, IL10RA, LAMA1, LIF, LILRB4, MADD, MAP4K1, MLL3, MMP19, MYBL2, NDRG4, NOP2, PIK3C3, PPP2R1B, PRKG1, PSAP, RERG, SCARB1, SH3BP2, SIK1, SLPI, STAMBP, TAGLN2, TMEFF2, TNFRSF1A, TNFRSF21, TP53I11
	Cell death and survival	21	BAG1, BNIP3L, CASP3, CYCS, E2F3, GNAS,GSK3A, HIF1A, HK2, ID1, ID2, IGF1R, NFKBIA, TXNIP, WTAP	ACTC1, EEF1A2, HLA-B, HOXB4, LIF, TNFRSF1A
	Cell cycle	41	ANXA2, ATF3, BCR, BRCC3, CDK6, CHKA, DUSP1, E2F3, EGR1, FOS, GNL3, GORASP2, HMGN1, ID1, ID2, IGF1R, ILKAP, KLF6, KPNA2, NAE1, NFKBIA, NPM1, POLA1, PRNP, PTGES3, RGCC, RPL7A, RPS6, SRSF2,TCP1, TXNIP, YWHAQ	BRSK2, EIF2AK2, IGFBP3, LIF, MYBL2, POLR2A, PSAP
Physiological system development and function	Hematologic system development and function	42	AES, AHNAK, BCLAF1, BCR, C1QBP, CASP3, CD69, CDK6, CXCL2, DUSP1, EGR1, FOS, HIF1A, HLA-DQB1, HOXA9, HPGDS, ID2, IKZF2, IMPDH1, INPP5D, KLF2, KLF9, MLLT3, NFKBIA, NPM1, NUP98, S1PR1, SATB1, TRAF6, TXNIP	AXL, EIF2AK2, GAPT, GPR183, HOXB7, IL10RA, LILRB4, MAP4K1, SH3BP2, SLPI, TNFRSF1A, TNFRSF21
	Hematopoiesis	14	ADAR, BCR, EGR1, HOXA9, ID1, NFKBIA, NUP98, RCOR1 MAP4K1, SLC37A4	AXL, EIF2AK2, HOXB4, LFNG,

Differentially expressed genes at all time-points analyzed after G-CSF mobilization relative to expression in non-treated samples, and biological processes in which they are implicated. Statistical significance: $P<0.05$.

miRNA expression of mobilized HPC in the long-term. It is already known that the mechanism of action of G-CSF is rather fast in terms of mobilization; in fact, the maximum release of HPC into peripheral blood appears only 5 days after the administration of G-CSF.[21] However, in our study, we observed that G-CSF produces changes much later among HPC and, remarkably, most of these changes persist for a long period of time. In this regard, we found that G-CSF modifies the gene and miRNA expression patterns of HPC even 1 year after its administration.

G-CSF induced the over-expression of most miRNA at all the different time-points analyzed. Moreover, the levels of expression of six of these miRNA (miR182, miR21, miR339-3p, miR483-5p, miR500 and miR576-3p) remained above the levels of their respective controls for at least 1 year after exposure to G-CSF. Among all of them, miR21, miR182 and miR339-3p may be the most relevant since they play a role as onco-miRNA. Thus, each of these miRNA regulates the expression of hundreds of genes and also targets several tumor suppressor genes, inhibiting their expression.[22-25] miR21 is involved in the control of angiogenesis, apoptosis, cell cycle, proliferation, stemness

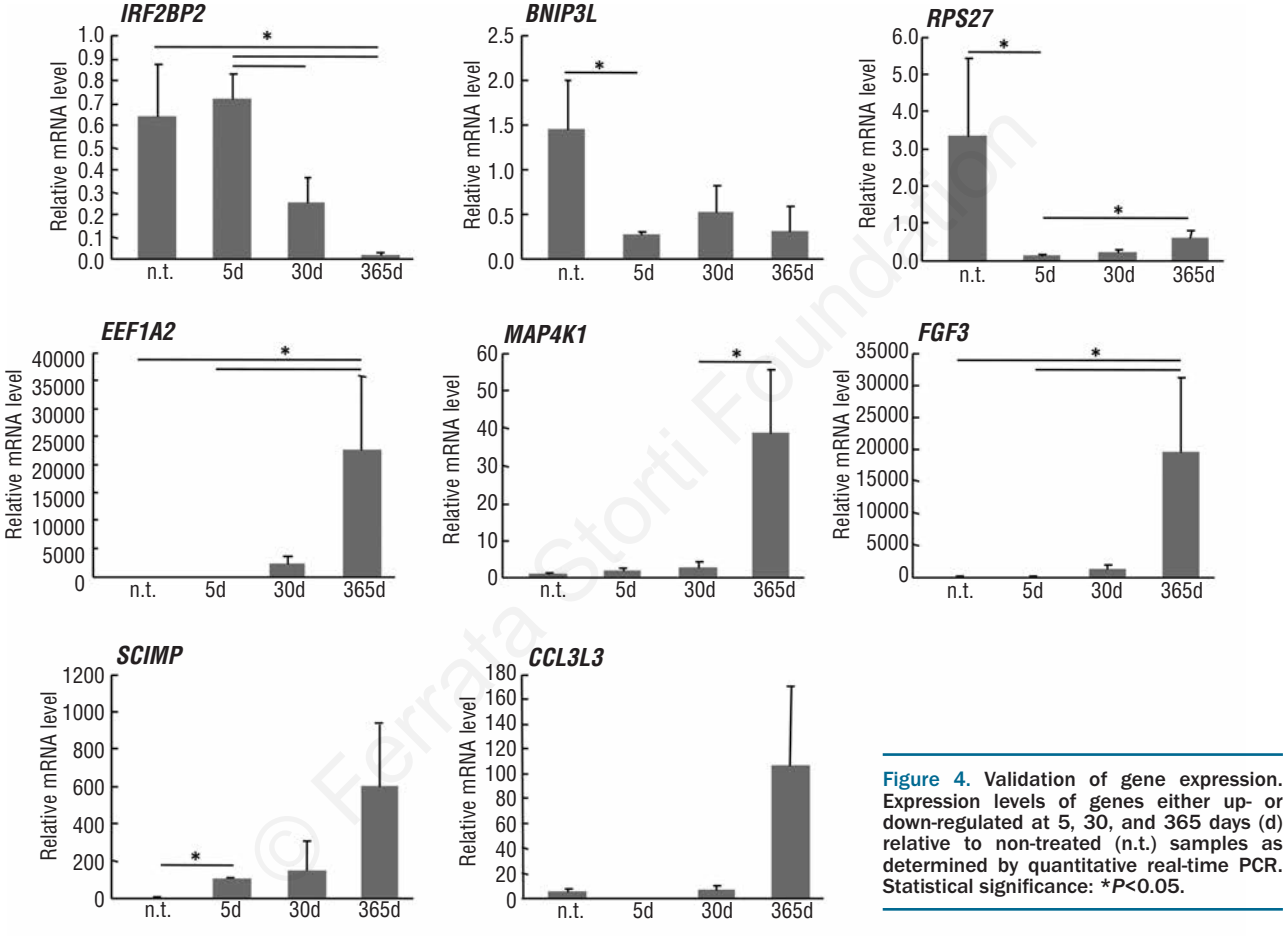

Figure 4. Validation of gene expression. Expression levels of genes either up- or down-regulated at 5, 30, and 365 days (d) relative to non-treated (n.t.) samples as determined by quantitative real-time PCR. Statistical significance: *$P<0.05$.

Table 3. Target genes.

miRNA	Target Genes
hsa-miR-182	ADSS, AHNAK, C20orf24, C6orf106, CBX5, CD69, CDK6, CDV3, CEP135, CITED2, CSNK1E, CYCS, DAZAP2, DNAJB6, DNAJB9, EEF1B2, FBXO33, GMFB, GNE, GNL3, GSK3A, HK2, HOXA9, IGF1R, INTS10, IRF2BP2, KDELR1, KDM6A, MAP1LC3B, MCFD2, MPP1, MY09A, NAA30, NAP1L1, NPM1, NUP43, PMPCB, PPP1R2, PSMD3, PSMF1, RAB34, RAD23B, RANBP2, RGPD8, SH3BGRL, SYNCRIP, TAPT1, TKT, TMEM230, WHSC1L1, WIPI2, ZNF706
hsa-miR-21	AMBRA1, ARMCX1, ARMCX5, ATP2A2, ATXN10, BDH2, BRCC3, CD69, CDK6, DNAJB9, E2F3, FAM156A/FAM156B, GNL3, IRF2BP2, KLF6, LARP1B, LTV1, MATR3, MBNL1, PURB, RAB21, RBMS1, REPS1, RNF103, RPS15, RSRC2, SATB1, SCRN1, STK40, TNRC6B, TSPAN3, UQCRB, WHSC1L1, YOD1, YPEL5
hsa-miR-339-3p	BDH2, CLASRP, DNAJB6, ID1, IGF1R, POLR3F
hsa-miR483-5p	AIPNL, BNIP3L,CDKN2, CXXC5,GNE, HYPK, IDS, IER2, KLF9, RAD23B, REPS1, RPL31, STK40
hsa-miR-500	ATP5F1
hsa-miR-576-3p	ARL2BP, ATP2A2, ATP6V0A1, BCLAF1, BNIP3L, BRCC3, BZW1, CBX5, CDV3, HS1BP3, KDM6A, KLF9, LAPTMYA, MED26, MMGT1, MPP1, MRPL43, PCNP, PGAM1, RCOR1, RPL37, RPS27, SRSF2, YWHAQ

Differentially expressed genes after G-CSF treatment regulated by the differentially expressed miRNA. All these genes were down-regulated in our analysis.

and immune response[26,27] and is frequently over-expressed in human cancers such as breast cancer, glioma, colorectal cancer, and hepatocellular carcinoma as well as hematologic malignancies.[28] miR182 and miR339-3p regulate cell growth, proliferation and cell cycle[29-31] and their over-expression has also been described in several hematologic diseases.[32] miR483-5p, miR500 and miR576-3p are involved in the processes of angiogenesis, cell cycle and immune response.[33] Our data raise the questions of whether the sustained over-expression of these miRNA induced by G-CSF could lead to modifications in any of these biological processes and whether or not this has any clinical implication.

On the other hand, G-CSF produced changes in protein-encoding gene expression levels at all time-points analyzed during the follow up. In our study we identified 617 genes with a greater modification in their expression levels after treatment. These genes are involved in several biological processes such as cancer, gene expression, protein synthesis, nucleic acid metabolism, cellular growth and proliferation, cell death and survival, hematopoiesis and hematologic system development and function. Of note, among all of them we found some interesting genes related to hematologic diseases; 11 of these genes were down-regulated after G-CSF mobilization (*BCR*, *CASP3*, *CXCL2*, *EGR1*, *FOS*, *HIF1A*, *HOXA9*, *NFKBIA*, *NPM1*, *NUP98* and *TXNIP*) and three were up-regulated (*AXL*, *EIF2AK2* and *MAP4K1*). BCR inhibits BCR-ABL oncogenic effects in chronic myeloid leukemia,[34] and it also participates in the regulation of cell cycle and gene expression. *CASP3* plays an important role in apoptosis and it is used as a prognostic marker for hematologic diseases such as chronic myeloid leukemia and B-cell lymphoma.[35] *CXCL2* encodes for a chemokine involved in neutrophil proliferation and migration during an immune response.[36] *EGR1* is a cancer suppressor gene that participates in cell differentation and mitogenesis.[37] *FOS* encodes for a regulator of cell proliferation, differentiation, and transformation. In some cases, expression of the *FOS* gene has also been associated with apoptotic cell death.[38] The *HIF1A* encoded protein functions as a regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes, including those involved in energy metabolism, angiogenesis, apoptosis, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia.[39] *HOXA9* is a homeobox gene encoding for a DNA-binding transcription factor which may regulate gene expression, differentiation, leukemogenesis and hematopoiesis.[40] *NFKBIA* encodes for a member of the NF-kappa-B inhibitor family and it is involved in inflammatory responses and tumor growth upon down-regulation.[41] *NPM1* is involved in several processes including regulation of the ARF/p53 pathway and tumor progression.[42] *NUP98* is a potential tumor suppressor gene found to be rearranged with many other genes in human hematologic malignancies.[43] *TXNIP* is also a tumor suppressor gene and it plays a pivotal role in the maintenance of hematopoietic cells.[44] *AXL* encodes for a tyrosine kinase receptor involved in several cellular functions including growth, migration, aggregation and anti-inflammation in multiple cell types.[45] The activated form of the *EIF2AK2* encoded protein can inhibit protein synthesis giving rise to various diseases[46] and *MAP4K1* is principally expressed in hematopoietic cells and is known to regulate stress responses, apoptosis and cell proliferation in cancer cells.[47]

Interestingly, among the 617 genes identified in our study, 109 were targets of the six miRNA over-expressed from day 5 to 1 year post-mobilization. To note, all these 109 genes were found to be down-regulated in our analysis (Table 3). Based on the information contained in the aforementioned databases, it is conceivable that the down-regulation of these 109 genes could be related to the over-expression of their corresponding regulatory miRNA.

Finally, when we validated some of the significant genes by quantitative real-time PCR, we confirmed that the G-CSF produced changes to the gene expression of HPC and, interestingly, some of these changes were even greater 1 year after the mobilization. Moreover, three of these validated genes were down-regulated after treatment and were targets of the differentially over-expressed miRNA.

From the information currently available in the literature,[5,6] we can assume that the administration of growth factors to healthy donors is safe; nevertheless, the potential effect in the long-term of these novel findings will require longer follow-up of larger series of donors.

In summary, we conclude that G-CSF modifies gene expression profiles and miRNA of HPC from healthy donors. These changes were observed from early time-points and most of them persisted for at least 1 year after exposure to the drug.

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