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

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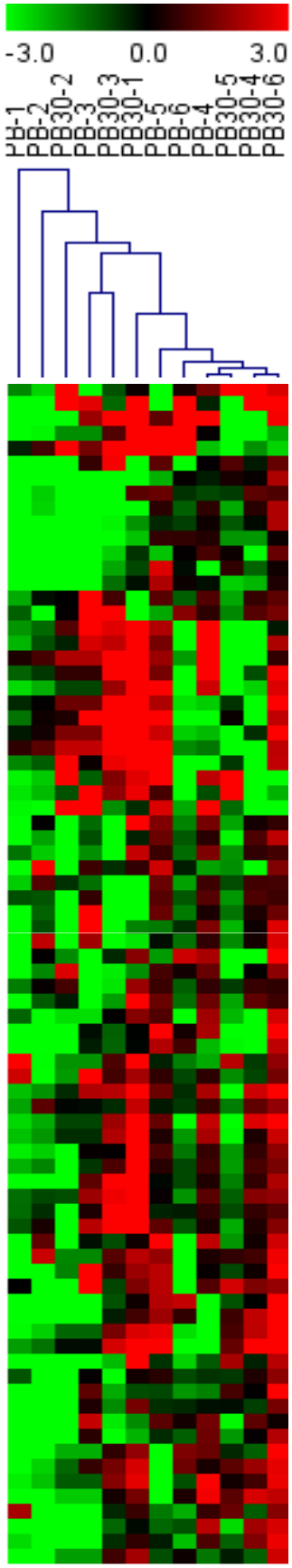
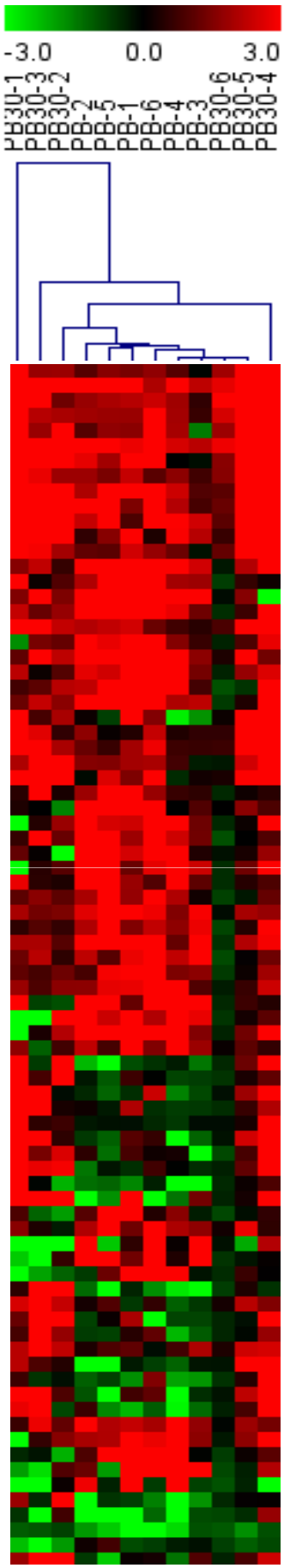


Supplementary Figure 1. Unsupervised hierarchical cluster analysis performed with the miRNAs from CD34+ cells from peripheral blood before (PB) and at 30 days (PB30) after a vehicle (saline solution) administration. Each numbered column represents an individual sample and each row represents a single miRNA. Panel contains a representative portion of the respective sets of miRNAs. Red and green color code indicates miRNA expression levels in logarithmic scale.



Supplementary Figure 2. Unsupervised hierarchical cluster analysis performed with the genes from CD34+ cells from peripheral blood before (PB) and at 30 days (PB30) after a vehicle (saline solution) administration. Each numbered column represents an individual sample and each row represents a single gene. Panel contains a representative portion of the respective sets of genes. Red and green color code indicates gene expression levels in logarithmic scale.

Supplemental table. Differentially expressed miRNAs after G-CSF mobilization

miRNAs	**Relative expression (log ratio)**																							
	PB-1	**PB-2**	**PB-3**	**PB-4**	**PB-5**	**PB-6**	**PB5-1**	**PB5-2**	**PB5-3**	**PB5-4**	**PB5-5**	**PB5-6**	**PB30-1**	**PB30-2**	**PB30-3**	**PB30-4**	**PB30-5**	**PB30-6**	**PB365-1**	**PB365-2**	**PB365-3**	**PB365-4**	**PB365-5**	**PB365-6**
hsa-miR-182	-1.42	-0.37	1.05	-2.53	-1.96	-0.30	1.52	3.99	-0.99	2.02	0.89	1.19	6.27	-0.03	1.70	7.39	6.62	-0.20	9.88	10.26	3.03	0.61	0.55	0.10
hsa-miR-21	-2.98	-2.17	-3.64	-1.99	-7.29	-4.99	1.59	-0.19	-0.47	2.78	0.00	0.71	1.58	-0.19	3.75	2.70	1.93	0.33	5.19	5.57	-1.65	4.08	-0.70	5.38
hsa-miR-339-3p	-2.78	-1.72	-0.30	-3.88	-2.93	-1.65	0.17	2.64	-1.06	4.48	3.86	1.53	4.92	-1.38	0.35	6.04	5.26	3.11	8.53	8.91	1.68	-0.74	3.23	-1.25
hsa-miR-483-5p	-1.42	-0.37	1.05	-2.53	-1.96	-0.30	1.52	3.99	3.64	4.18	7.67	5.99	6.27	-0.03	7.29	7.39	6.62	4.99	7.88	10.26	3.03	1.61	1.55	4.15
hsa-miR-500	-1.42	-0.03	1.05	-2.53	-1.96	-0.30	1.52	3.99	-0.60	1.85	1.04	1.19	6.27	0.91	1.70	7.39	6.62	-0.20	9.88	10.26	3.03	0.61	0.55	0.10
hsa-miR-576-3p	-1.42	-0.37	1.05	-2.53	-1.96	-0.30	7.33	3.99	-0.08	2.71	0.89	1.19	6.27	2.40	1.70	7.39	9.76	-0.20	7.78	9.36	3.03	1.70	0.55	2.01

Expression levels of the differentially expressed miRNAs at all time-points analyzed after G-CSF mobilization relative to non-treated samples.

Statistical significance: $p < 0.05$

Supplemental Methods

Samples

CD34+ progenitor cells from peripheral blood (PB) of 6 healthy donors were collected before and at 5, 30 and 365 days after the mobilization with G-CSF. The mobilization regimen was based on the administration of 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 HPCs

Mononuclear cells were collected from all samples by density gradient centrifugation with Ficoll-Paque solution (Amersham Biosciences, Uppsala). 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). Further, for a higher purity of isolation, CD34+ cells were sorted by flow cytometry (MoFlo, Beckman Coulter). For this purpose, cells were incubated with the monoclonal antibodies CD34-PE and CD45-FITC (Becton Dickinson, San Jose, CA) for 20 minutes in darkness and at room temperature. Populations were selected based on the intensity of antibodies as well as forward and side scattered components (FSC and SSC). Dead cells were discarded before separation. The purity of the isolated CD34+ cells was higher 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 was verified by a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA); only the samples with RNA integrity number (RIN) higher than 7.5 were used for further analyses of miRNA and gene expression profiling.

miRNA expression

The expression profile of 384 miRNAs was analyzed in all samples. Total RNA (150 ng) was reverse-transcribed using the miRNA TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was loaded on to the TaqMan Human MicroRNA v2.0 Arrays (Applied Biosystems, Foster City, CA) which were subsequently analyzed on a 7900 HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA). The SDS 2.3 and RQ Manager 1.2 software (both Applied Biosystems, Foster City, CA) were used for the arrays analysis. Undetectable miRNAs were excluded for further analyses. Data were normalized using the average of the endogenous small-nucleolar RNU48 and the non-coding small nuclear U6, both included in the array, and a group of samples of CD34+ cells from PB was used as control group. The expression levels of miRNAs were obtained by the $2^{-\Delta\Delta CT}$ method.

Gene expression

We analyzed the gene expression profiling of 45000 genes in the same samples using the Whole Human Genome Oligo microarray kit 4x44K (Agilent Technologies, Santa Clara, CA). Total RNA (200 ng) was reverse-transcribed to cRNA and labeled with the two Color Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA). The quality and integrity of the cRNA was verified by a Bioanalyzer 2100 (Agilent Technologies, Santa

Clara, CA). Every analyzed sample was Cyanine3-labelled and hybridized against a pool of Cyanine5-labelled RNA of CD34+ cells from PB as reference group. The microarrays were scanned in a GenePix reader (Molecular Devices, Sunnyvale, CA).

Validation of significant genes

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). Data were normalized to the housekeeping gene *ACTB* and the same group of samples of CD34+ cells from PB used for the hybridization experiments was used as control. The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Unsupervised hierarchical clusters of gene and miRNA expression data were performed using the average linkage and the Euclidean distance. To identify the genes and miRNAs differentially expressed in CD34+ cells before and at the different time-points after the G-CSF administration we applied non-parametric Mann-Whitney test. To obtain positive and negative expression values data were transformed to logarithmic scale. All analyses were performed using the Multi-experiment Viewer 4.7.1 software. The function of the genes and miRNAs of interest was determined from different databases available online (miRbase, Gene Ontology, TargetScan Human 6.2, Ingenuity Pathways Analysis).