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The granulocyte colony-stimulating factor produces long-term changes on gene and miRNA expression profiles in CD34+ cells from healthy donors

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The granulocyte colony-stimulating factor produces long-term changes on gene and miRNA expression profiles in CD34+ cells from healthy donors

Running heads: G-CSF effect in HPCs expression profiles

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Abstract

The Granulocyte-colony stimulating factor is the most commonly used cytokine for the mobilization of hematopoietic progenitor cells from healthy donors for the allogeneic stem cell transplantation. Although the administration of this cytokine is considered safe, the knowledge about its long-term effects, especially in hematopoietic progenitor cells, is limited. With this background, the aim of our study was to analyze whether or not the Granulocyte-colony stimulating factor might induce changes on gene and miRNA expression profiles in hematopoietic progenitor cells from healthy donors, and to determine whether or not these changes persist at the long-term. For this purpose, we analyzed the whole genome expression profile and the expression of 384 miRNAs in CD34+ cells isolated from peripheral blood of 6 healthy donors, before the mobilization and at 5, 30 and 365 days post-mobilization with the Granulocyte-colony stimulating factor. Six miRNAs were differentially expressed at all time points analyzed after mobilization treatment as compared to samples obtained before exposure to the drug. In addition, 2424 genes were also differentially expressed for at least 1 year post-mobilization. Of interest, 109 of these genes are targets of the differentially expressed miRNAs also identified in this study. These data strongly suggest that the Granulocyte-colony stimulating factor modifies gene and miRNA expression profiles in hematopoietic progenitor cells from healthy donors. Remarkably, some changes are observed from early time-points and persist 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 (HPCs) from bone marrow to blood stream by growth factors for hematopoietic transplantation was introduced into the clinical practice in the decade of 80s.[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 the normal population.[5,6] Furthermore, in comparison to bone marrow donation, the use of HPCs from peripheral blood is related to a better donor's quality of life early post-donation.[7] At the molecular level, G-CSF induces the release of proteolytic enzymes by neutrophils to the extravascular compartment of the bone marrow. These enzymes degrade and inactivate the linkage proteins between HPCs and the bone marrow stroma releasing the HPCs into the peripheral blood. Among the receptors involved in the interaction between HPCs and stroma the most important is the axis SDF-1/CXCR4.[8-13] In addition, it is known that G-CSF produces a decrease in the expression of stromal cell derived factor (SDF-1) in the marrow stroma.[14] Nevertheless, there is not much information about the effects of the G-CSF on the expression of other genes within HPCs.

Several studies have described that miRNAs may also play an important role in the mobilization of the HPCs. Indeed, HPCs have different miRNA expression profiles depending on whether they are mobilized with G-CSF or Plerixafor.[15,16] miRNAs are involved in different biological processes including development, differentiation,

proliferation and cell death.[17] Accordingly, changes in the expression of miRNAs associated to the drug-induced mobilization of HPCs might lead to changes in gene expression. In this regard, it has been described that G-CSF induces changes on both, gene and miRNA expression profiles, in leukocytes for up to 9 months post-mobilization.[18-20] However, its effects at the long-term on HPCs, need to be analyzed.

With this background, we hypothesized that the G-CSF could modify gene and miRNA expression profiling of HPCs resulting in changes that could affect their biological features. Therefore, the aim of this study was to analyze with high throughput techniques whether G-CSF induces changes on gene and miRNA expression profiles in HPCs from healthy donors, and to determine whether these changes in the expression signatures persist at the long-term or return to the original status.

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 (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 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) and, after magnetic enrichment, CD34+ cells were sorted by flow cytometry. 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).

miRNA and gene expression

The expression profile of 384 miRNAs was analyzed in all samples using TaqMan Human MicroRNA v2.0 Arrays (Applied Biosystems, Foster City, CA) which were 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 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 45000 genes was analyzed in the same samples using the Whole Human Genome Oligo microarray kit 4x44K (Agilent Technologies, Santa Clara, CA). The microarrays were scanned in a GenePix reader (Molecular Devices, Sunnyvale, CA).

In both types of expression analysis, samples from non-mobilized CD34+ cells were used as reference group.

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 group of samples from non-mobilized CD34+ cells used 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, Ingenuity Pathways Analysis).

Results

Effect of G-CSF on miRNA expression of HPCs

miRNA expression profiles were determined in CD34+ cells from PB before and at different time-points after the mobilization with G-CSF. Seventy two out of 384 miRNAs were undetectable across all samples and were excluded from further analyses. We performed unsupervised paired hierarchical clusters comparing the expression of the miRNAs 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 on miRNA expression pattern until one year after mobilization. To identify those miRNAs differentially expressed between the different groups, a non-parametric Mann-Whitney test was applied. All miRNAs with a p value < 0.05 were considered significant. Figure 1 shows paired hierarchical clusters including only differentially expressed miRNAs.

At day 5 after treatment, we identified 15 differentially expressed miRNAs, 12 of them being over-expressed and 3 under-expressed as compared to non-mobilized CD34+ cells (Figure 1A).

At day 30, we found 179 differentially expressed miRNAs, 177 over-expressed and 2 under-expressed (Figure 1B). Out of these, 9 miRNAs were also over-expressed at day 5 post-mobilization, whereas 168 miRNAs appeared over-expressed for first time at day 30 of the mobilization.

One year after G-CSF administration, we identified 155 differentially expressed miRNAs, 130 of them were over-expressed and 25 under-expressed as

compared to the control (Figure 1C). Among all of these miRNAs, 128 were also over-expressed at day 30 and maintained their expression levels one year after G-CSF, while 6 miRNAs (miR182, miR21, miR339-3p, miR483-5p, miR500 and miR576-3p) showed over-expression since the start of the treatment up to one year later as compared to the controls (Figure 2). These miRNAs are mainly involved in processes such as cell cycle, proliferation, angiogenesis and immune response (Table 1). Overall, we found that G-CSF induce mostly over-expression of miRNAs in mobilized HPCs. The highest number of over-expressed miRNAs is observed on day 30 after G-CSF administration and the same trend remain at least for one year (Figure 1D).

In addition, in order to check that the miRNA expression pattern of CD34+ cells from PB do not vary over time in normal conditions, we performed the same unsupervised hierarchical cluster comparing the miRNA expression profiles of CD34+ cells from PB of 6 control subjects, collected before and after 30 days of the administration of a vehicle (saline solution). As we expected there were not changes in the expression pattern of miRNAs of a subject over time (Supplementary Figure 1).

Effect of G-CSF on protein-encoding gene expression of HPCs

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 post-mobilization clearly identified two clusters which included the samples obtained before and after exposure to G-CSF, respectively. Interestingly, most of the differences on treated samples remained after one year post-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 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, 1848 were up-regulated and 3112 were down-regulated 30 days post-mobilization as compared to un-treated samples (Figure 3B). Of all these genes, 1899 appeared differentially expressed for first time at day 30 of the mobilization, 635 up-regulated and 1264 down-regulated.

Finally, upon comparing CD34+ cells before and after one year of mobilization, we found 4805 differentially expressed genes, out of them, 1969 were up-regulated and 2836 down-regulated (Figure 3C). In this case 2381 genes appeared differentially expressed at first time one year after G-CSF treatment, 1075 were up-regulated and 1306 down-regulated.

Among all of the differentially expressed genes, 2424 maintained their expression levels from day 5 to one year after the treatment with G-CSF; 894 of these genes were up-regulated and 1530 down-regulated as compared to 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, out of which 232 were up-regulated and 385 were down-regulated after mobilization, as compared to the control. The functional analysis of these genes by 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 the G-CSF alters the expression profiles of genes in mobilized HPCs. Similarly to miRNAs, the highest number of up- or down-regulated genes was observed after 30 days of treatment. Again, most of these changes persist at least one year after mobilization (Figure 3D).

Furthermore, we compared the gene expression patterns of CD34+ cells from PB of 6 control subjects, collected before and after 30 days of the administration of a vehicle (saline solution). Similarly as observed in miRNAs there was not variability in the time of gene expression levels in individual normal subjects (Supplementary Figure 2).

At last, in order to validate the high-throughput screening, we confirmed the expression of 8 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* are related with cell proliferation and immune response. *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 hematological system 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 one 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 miRNAs identified in the current study. These 3 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 with some hematological 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 miRNA expression of mobilized HPCs at 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 HPCs to peripheral blood appears only five days after G-CSF administration.[21] However, in our study, we observed that the G-CSF produces changes much later among HPCs and, remarkably, most of these changes persist for a long period of time. In this regard, we found that the G-CSF modifies the gene and miRNA expression patterns of HPCs even one year after its administration.

G-CSF induced the over-expression of most miRNAs at all the different time-points analyzed. Moreover, six of these miRNAs (miR182, miR21, miR339-3p, miR483-5p, miR500 and miR576-3p) maintained their expression levels above their respective controls at least one year after exposure to the 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 miRNAs regulates the expression of hundreds of genes and also target several tumor suppressor genes, inhibiting their expression.[22-25] miR21 is involved in the control of angiogenesis, apoptosis, cell cycle, proliferation, stemness 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 in hematological 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 hematological 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 question of whether the sustained over-expression of these miRNAs induced by G-CSF could lead to modifications in any of these biological processes and whether or not this might have any clinical implication.

On the other hand, G-CSF produced changes on protein-encoding gene expression levels at all time-points analyzed during the follow up. In our study we identified 617 genes with a higher 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 hematological system development and function. Of note, among all of them we found some interesting genes related to hematological diseases; 11 of these genes were down-regulated post G-CSF mobilization (*BCR*, *CASP3*, *CXCL2*, *EGR1*, *FOS*, *HIF1A*, *HOXA9*, *NFKBIA*, *NPM1*, *NUP98* and *TXNIP*) and 3 up-regulated (*AXL*, *EIF2AK2* and *MAP4K1*). *BCR* inhibits the 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 prognostic marker for hematological diseases such as chronic myeloid leukemia or 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 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 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 the 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 of them were targets of the 6 miRNAs over-expressed from day 5 to one 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 miRNAs.

Finally, when we validated some of the significant genes by quantitative real-time PCR, we could confirm that the G-CSF produced changes on the gene expression of HPCs and, interestingly, some of these changes were even higher after one year of the mobilization. Moreover, 3 of these validated genes were down-regulated after treatment and were targets of the differentially over-expressed miRNAs.

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

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

Authorship and disclosures

AB processed all samples, performed gene expression microarrays, miRNAs arrays, analyzed data and wrote the manuscript; BMA designed the research project and analyzed data; JIP and IAL analyzed data; CP and MC collected samples from donors; MVB critically reviewed the manuscript; AUI provided funding, designed the research project and critically reviewed the manuscript and JPS critically reviewed the research project and the manuscript.

The authors report no potential conflicts of interest.

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Tables

Table 1. Differentially expressed miRNAs after G-CSF mobilization

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

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
MOLECULAR AND CELLULAR FUNCTIONS	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

MOLECULAR AND CELLULAR FUNCTIONS	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
	Nucleic acid metabolism	19	ATP2A2, ATP5E, ATP5F1, ATP5O, ATP6V0C, CYCS, HIF1A, IMPDH1, MAP1LC3B, MSH6, NT5C2, SLC25A5, SOD1, TXNIP, WRN	ACTC1, AK4, ASK, PPP2R4

MOLECULAR AND CELLULAR FUNCTIONS	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,	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

MOLECULAR AND CELLULAR FUNCTIONS			YME1L1, YWHAQ, ZMIZ1, ZSCAN18	
	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	Hematological 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	AXL, EIF2AK2, HOXB4, LFNG, MAP4K1, SLC37A4

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

Table 3. Target genes

miRNAs	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 miRNAs. All these genes are down-regulated in our analysis.

Figure Legends

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

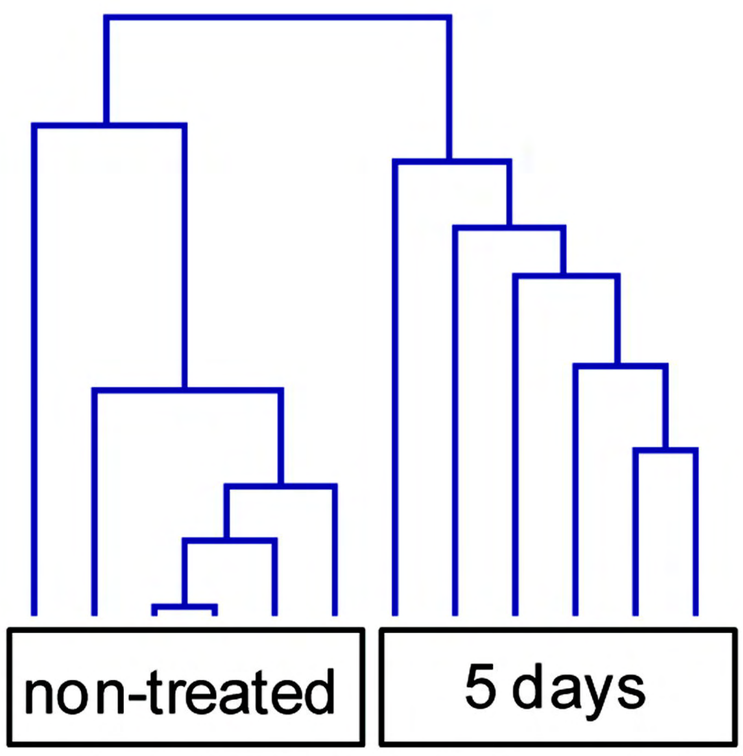
Figure 2. Expression levels of differentially expressed miRNAs at all the three time-points analyzed after G-CSF administration. Differences in miRNA expression at 5, 30, and 365 days (d) relative to non-treated (n.t.) samples. Statistical significance: *; $p < 0.05$.

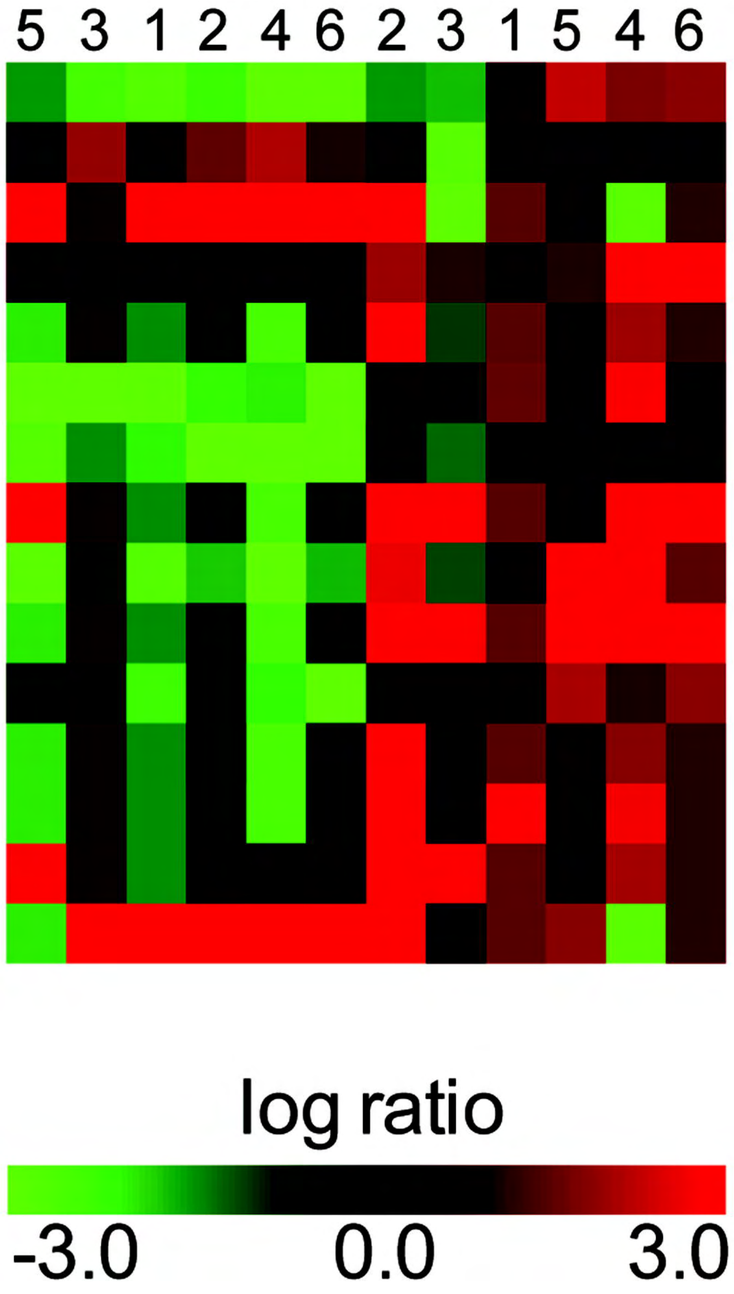
Figure 3. Unsupervised hierarchical cluster analysis of genes. The analysis was performed with the genes differentially expressed between CD34+ cells from non-treated samples and at 5 days (**A**), 30 days (**B**), and one year (**C**) after G-CSF administration respectively. Each numbered column represents an individual sample and each row represents a single gene. Panels contain representative portion of the respective sets of genes. Red and green color code indicates gene expression levels in logarithmic scale. Statistical analysis was performed using 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.

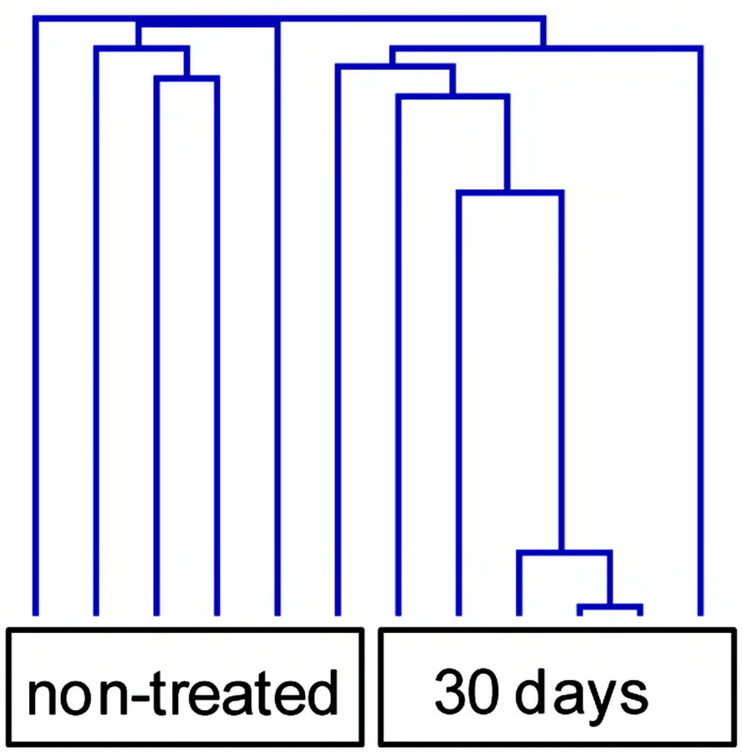
Figure 4. Validation of gene expression. Expression levels of genes either up- or down-regulated at 5, 30, and 365 days (d) relative to non-treated (n.t.) samples as determined by quantitative real-time PCR. Statistical significance: *; $p < 0.05$.

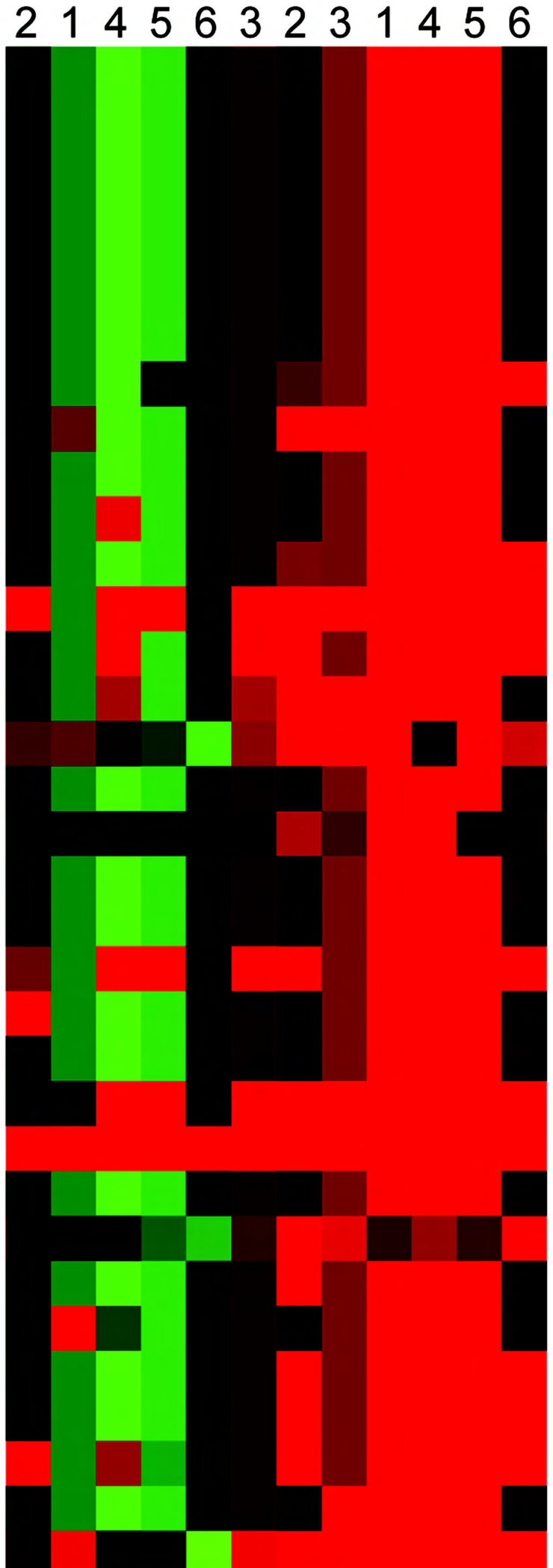
A


non-treated
5 days

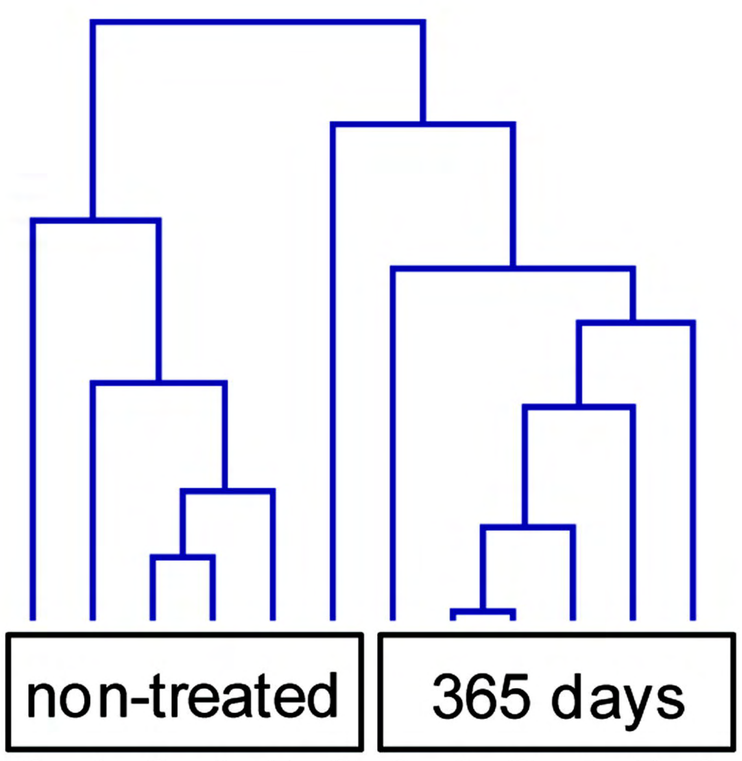

5 3 1 2 4 6 2 3 1 5 4 6

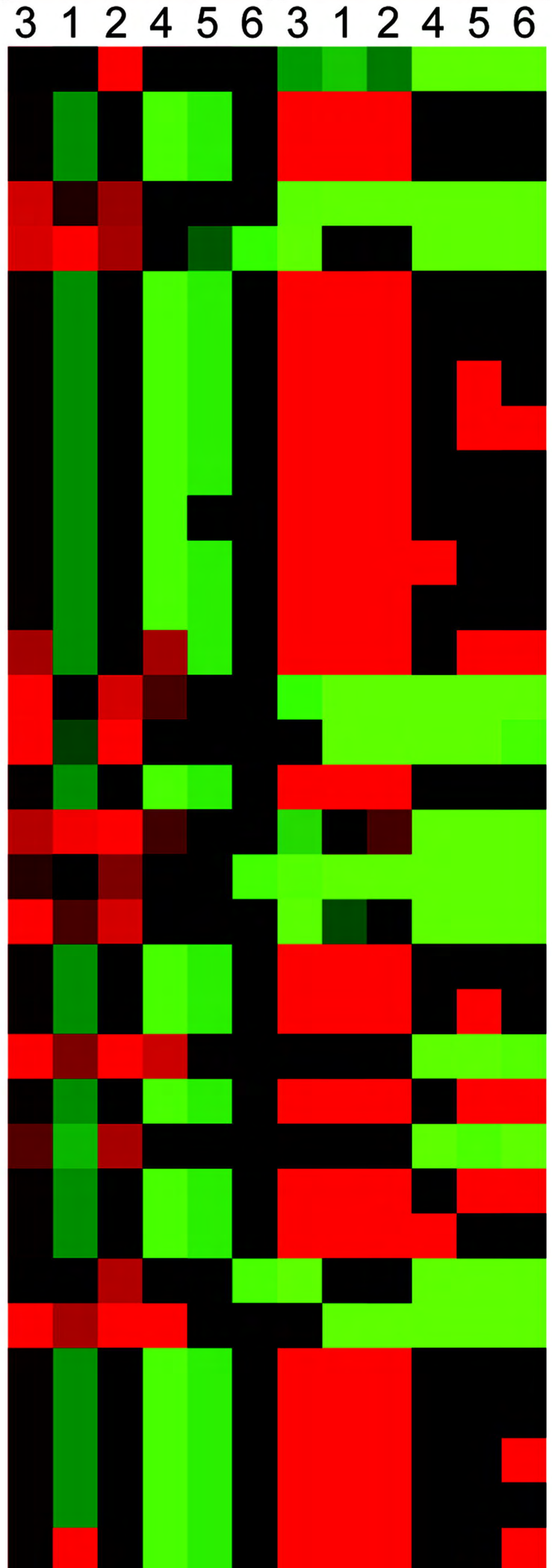
B


non-treated
30 days


2 1 4 5 6 3 2 3 1 4 5 6

C


non-treated
365 days


3 1 2 4 5 6 3 1 2 4 5 6

log ratio
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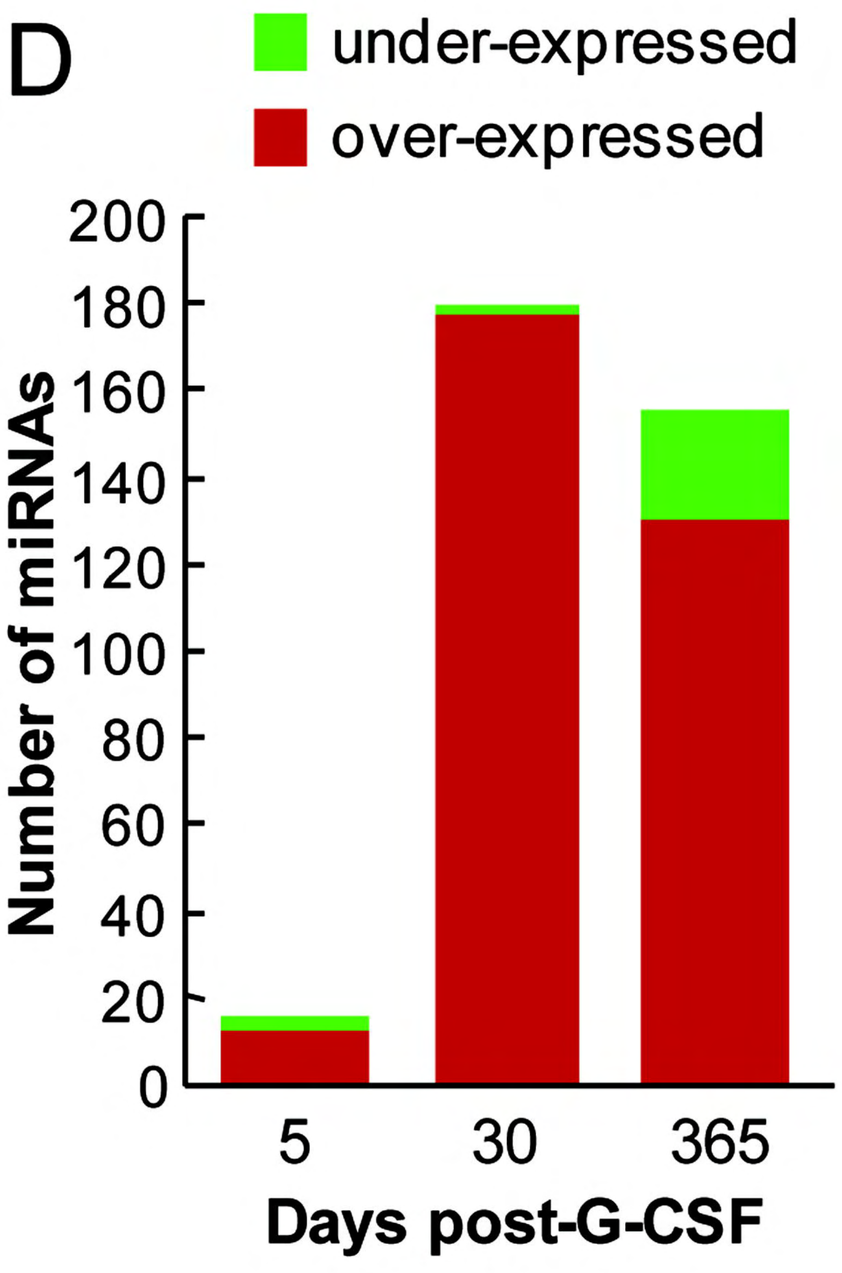
0.0
3.0

D


under-expressed
over-expressed
Number of miRNAs
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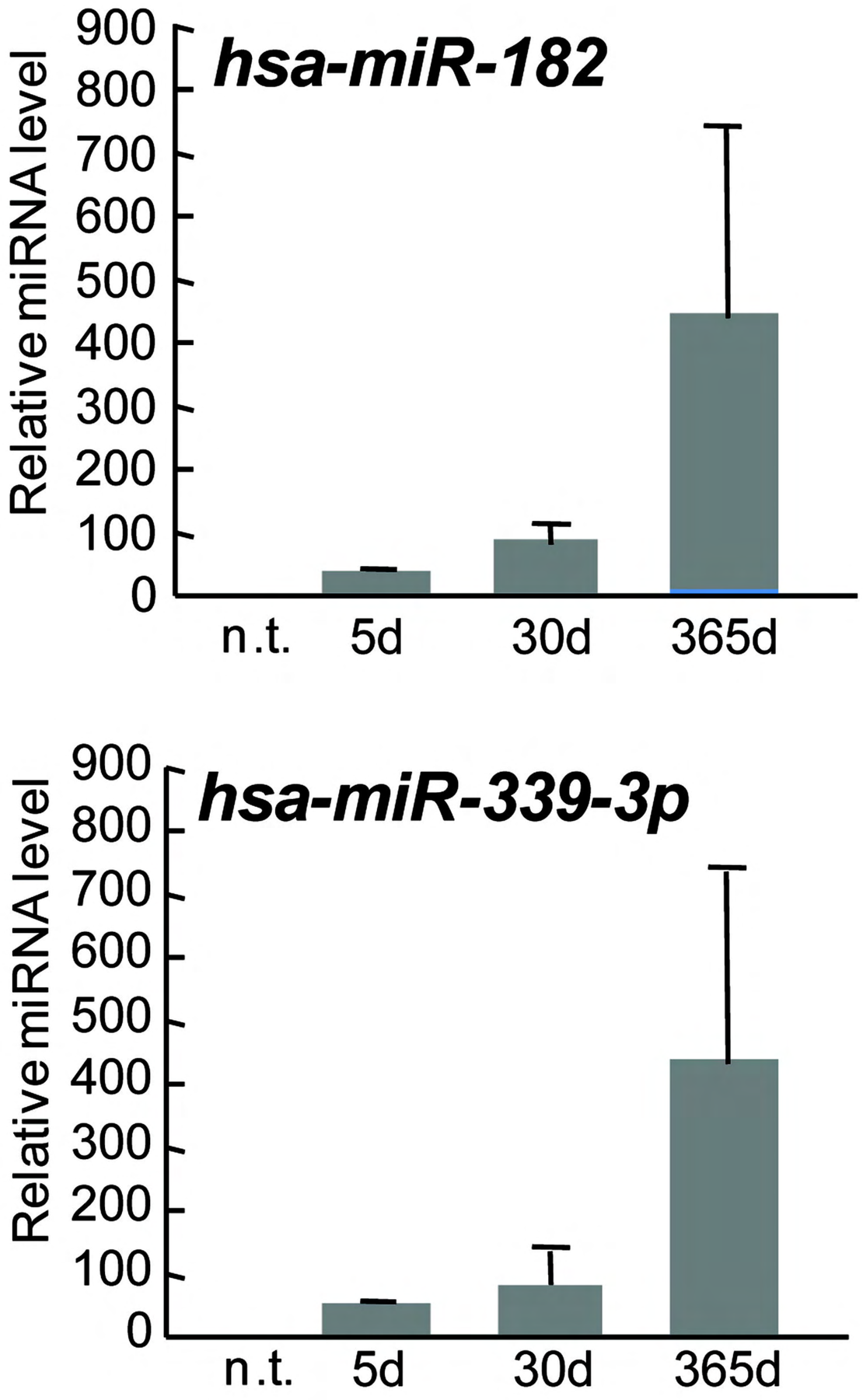
180
160
140
120
100
80
60
40
20
0
5
30
365
Days post-G-CSF


hsa-miR-182
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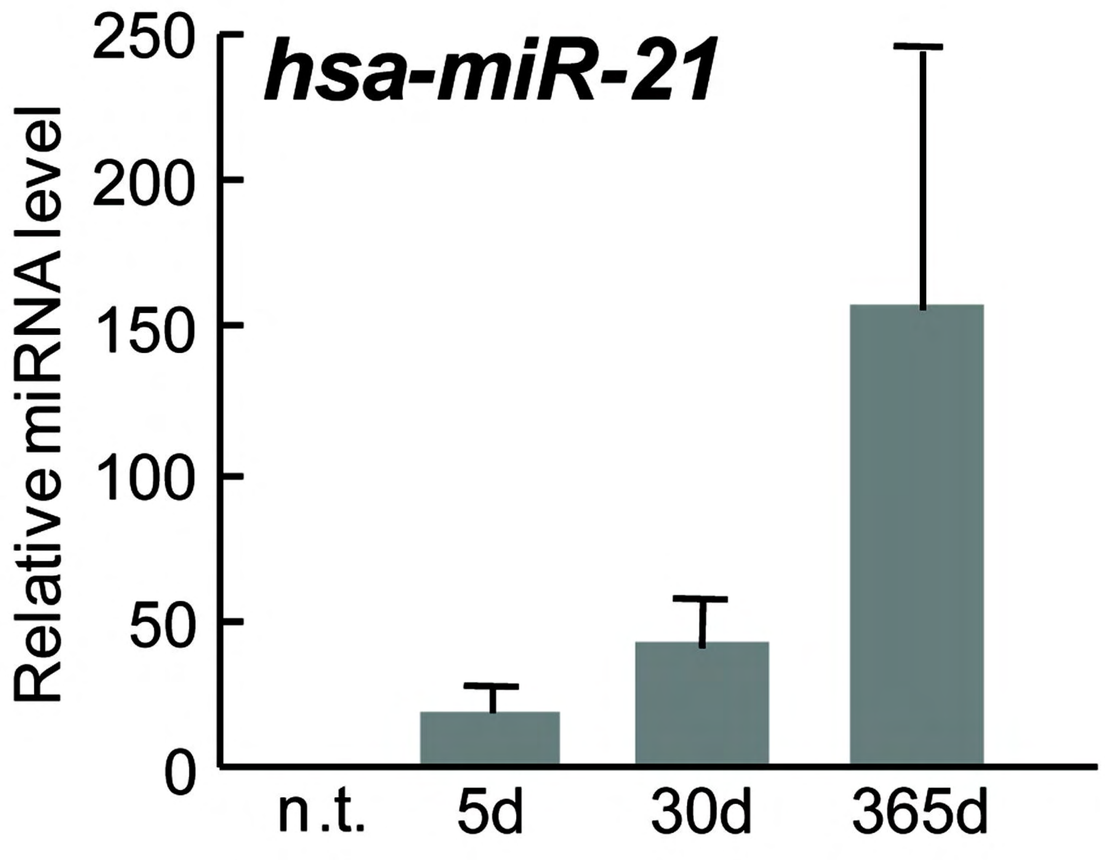
n.t.
5d
30d
365d


hsa-miR-21
Relative miRNA level
250
200
150
100
50
0
n.t.
5d
30d
365d

hsa-miR-339-3p
Relative miRNA level
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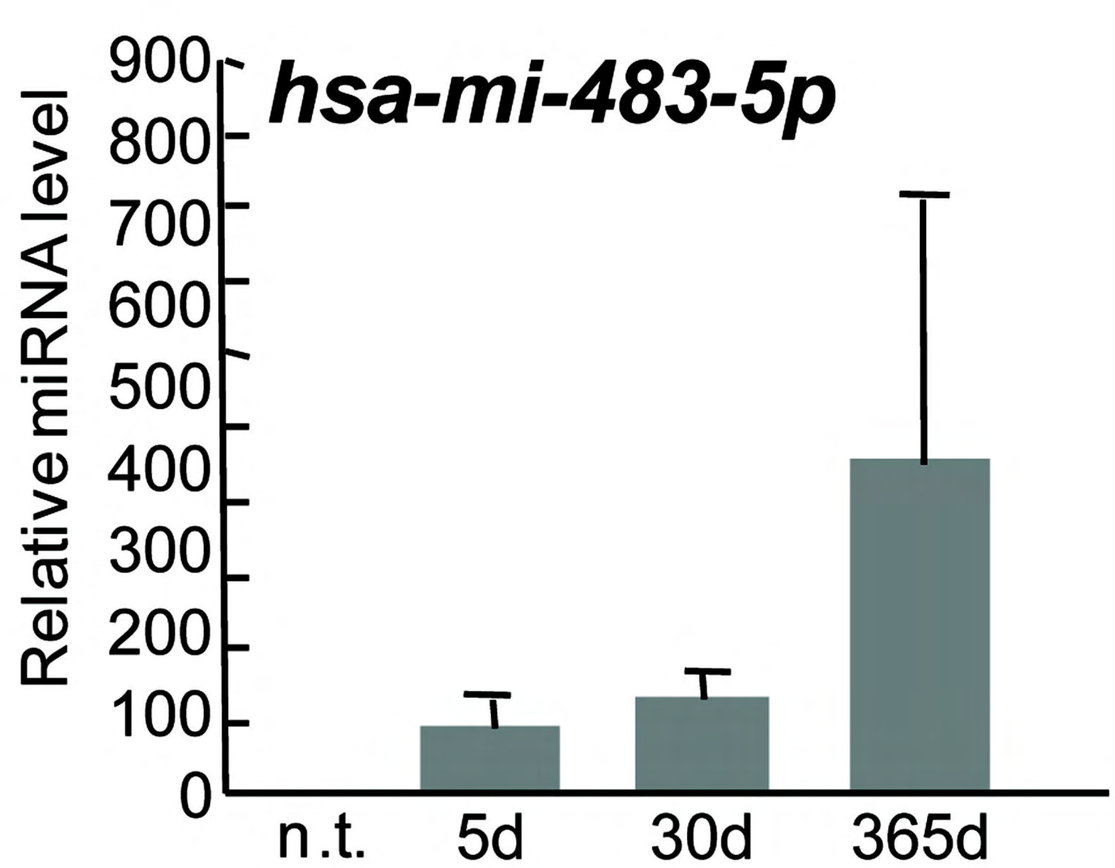
800
700
600
500
400
300
200
100
0
n.t.
5d
30d
365d


hsa-mi-483-5p
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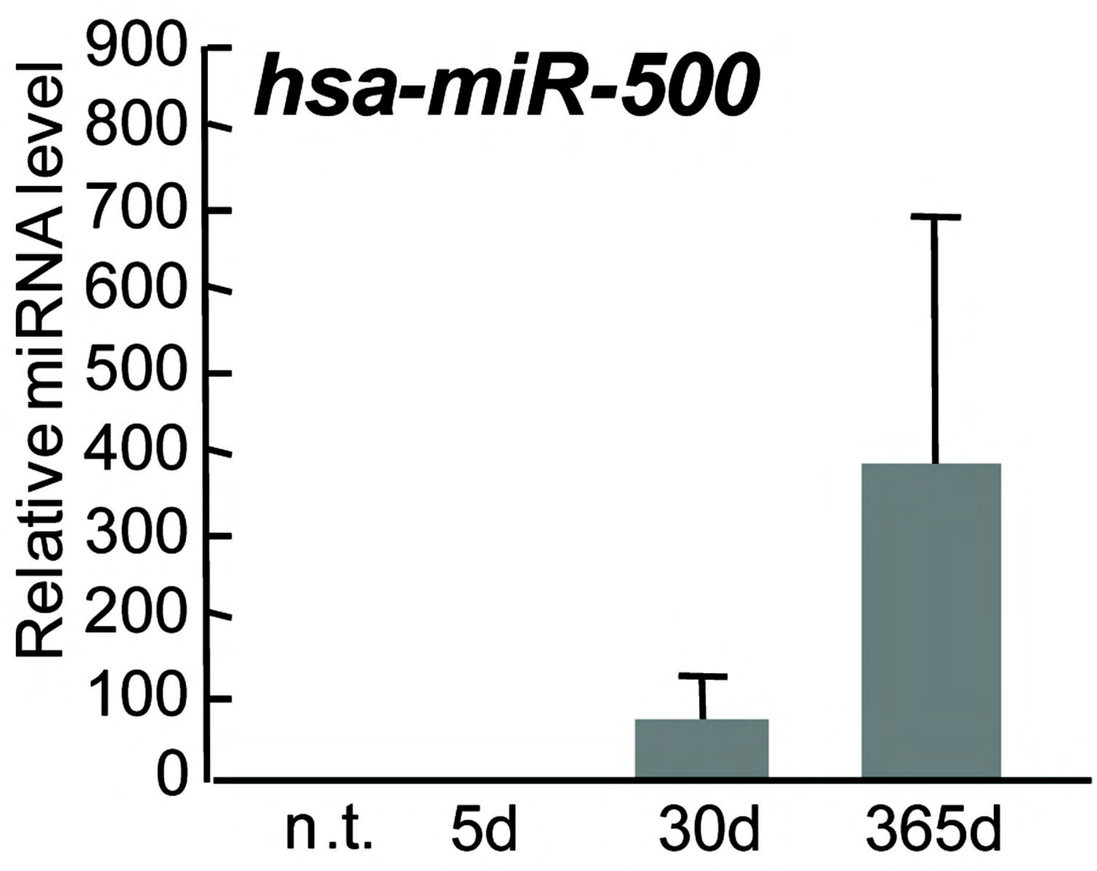
500
400
300
200
100
0
n.t.
5d
30d
365d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Relative miRNA level
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700
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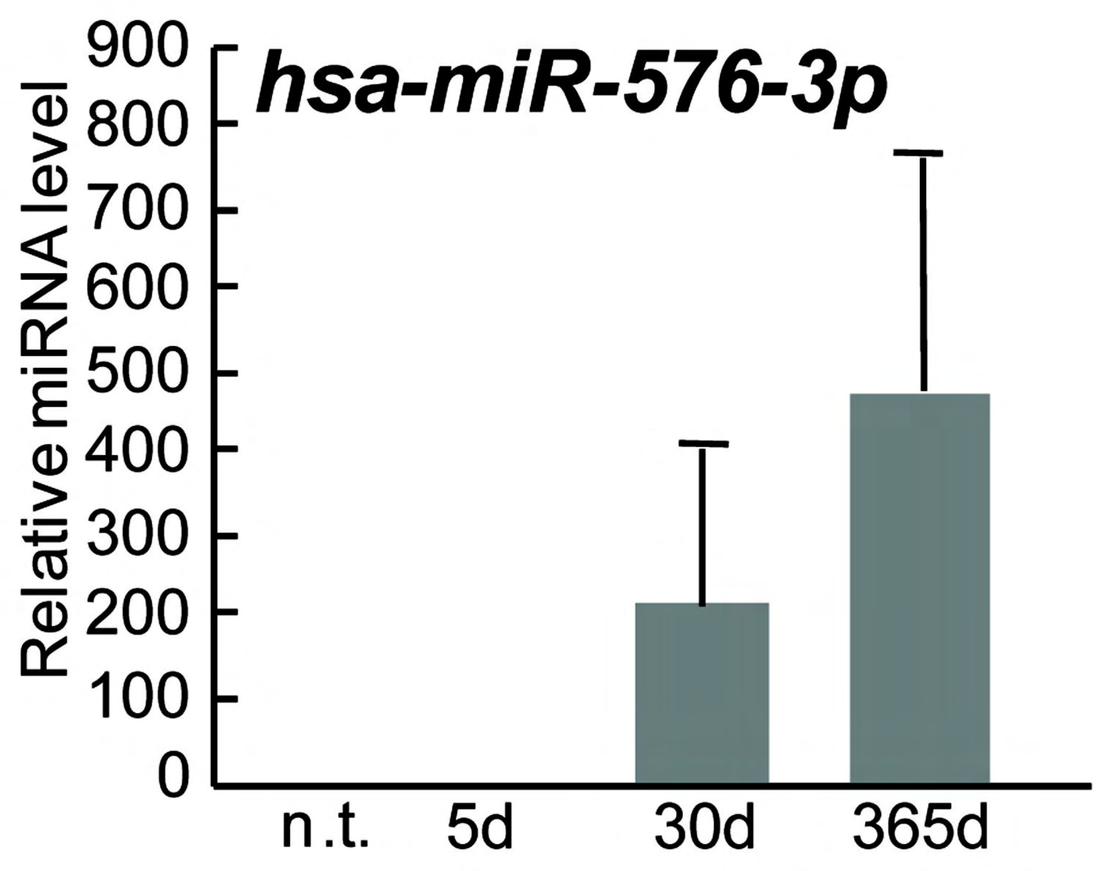
500
400
300
200
100
0
n.t.
5d
30d
365d


hsa-miR-576-3p
Relative miRNA level
900
800
700
600
500
400
300
200
100
0
n.t.
5d
30d
365d

A

non-treated | 5 days

3 1 2 4 5 6 1 2 3 4 5 6

B

non-treated | 30 days

1 2 3 4 5 6 3 1 4 2 5 6

C

non-treated | 365 days

2 1 4 3 5 6 4 6 1 5 2 3

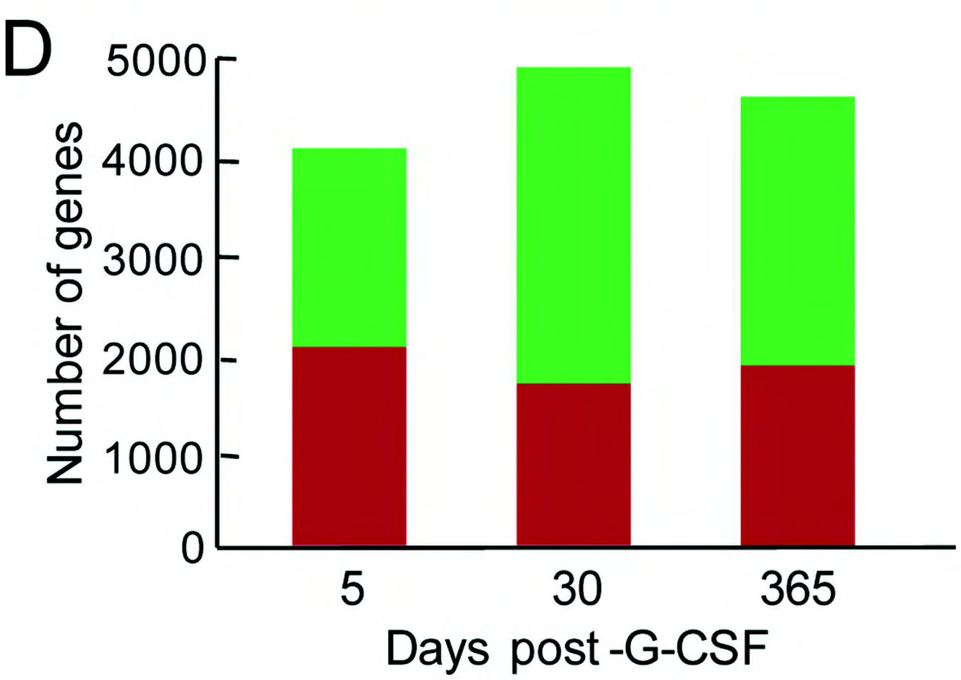
log ratio

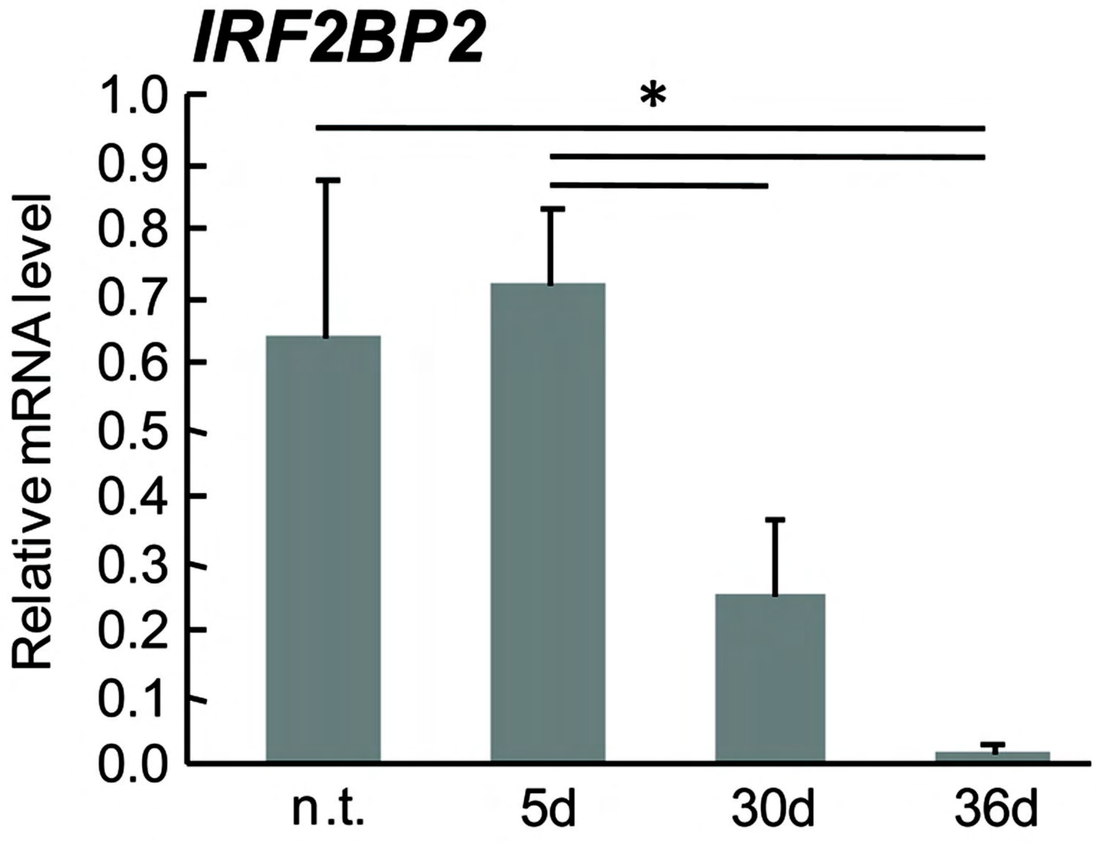
-3.0 0.0 3.0

down-regulated

up-regulated

D




IRF2BP2
Relative mRNA level
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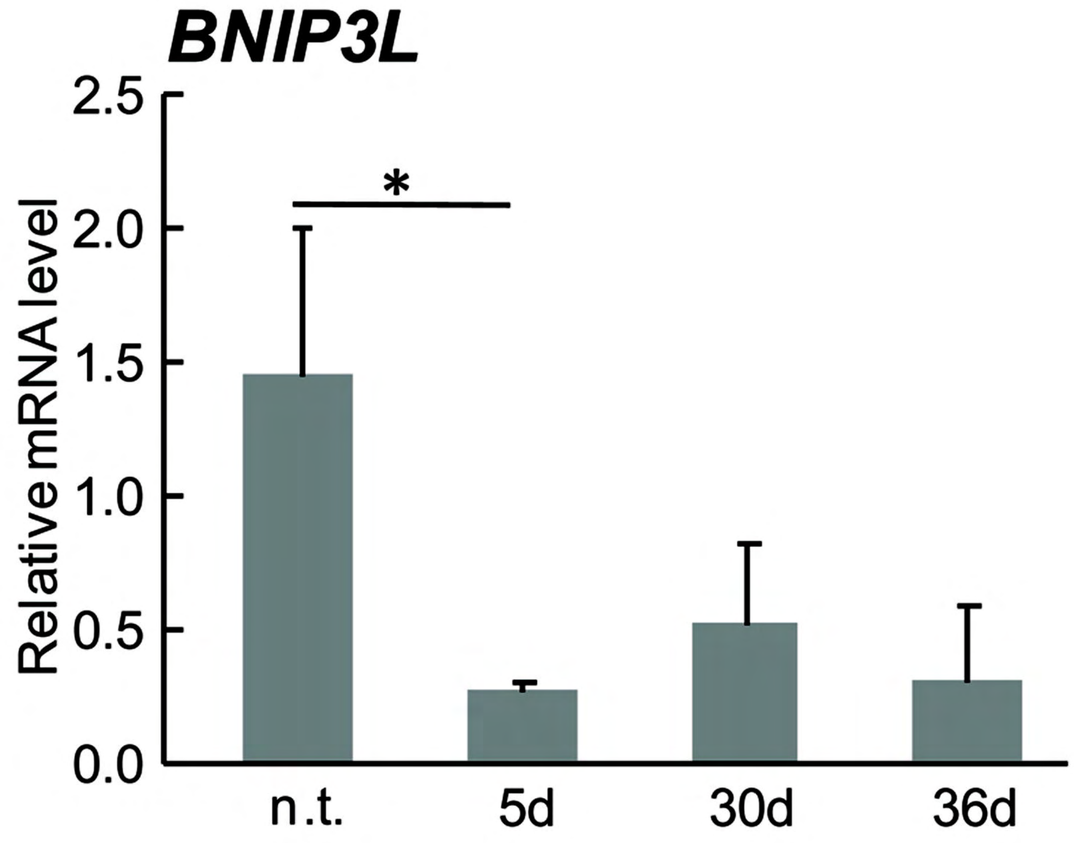
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
n.t.
5d
30d
36d


BNIP3L
Relative mRNA level
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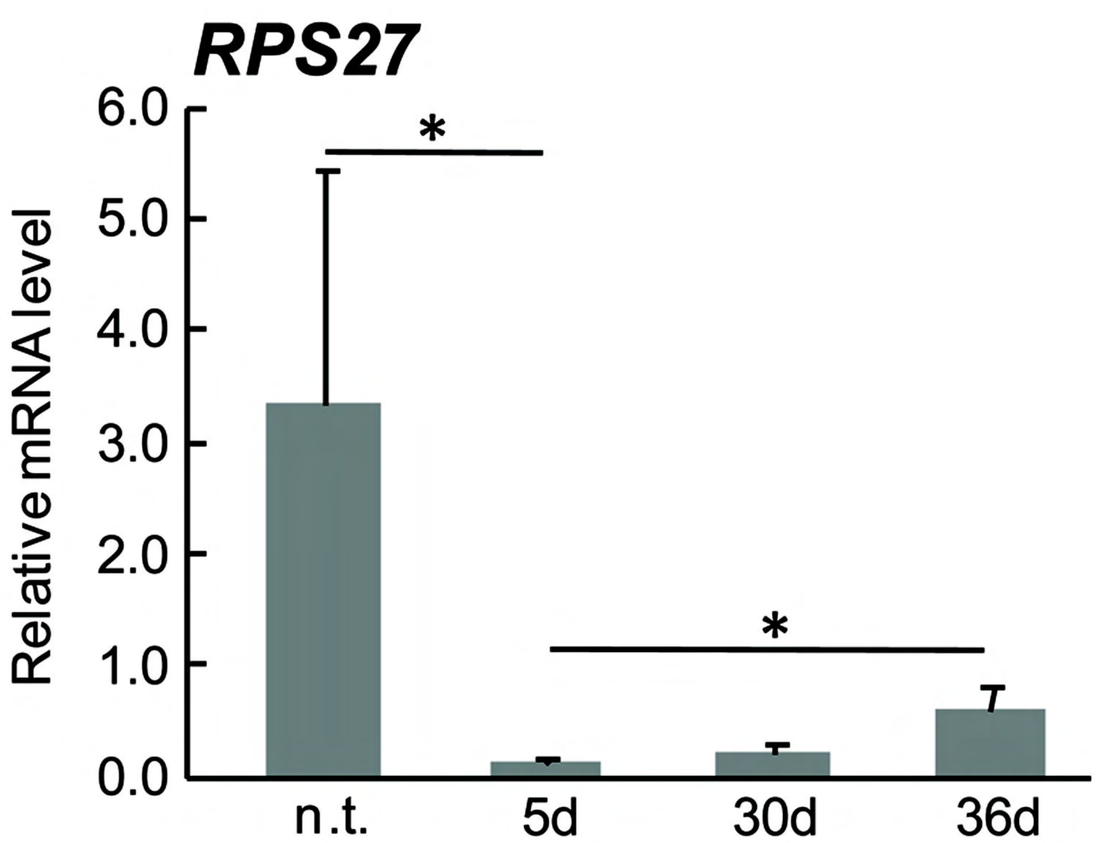
2.0
1.5
1.0
0.5
0.0
*
n.t.
5d
30d
36d


RPS27
Relative mRNA level
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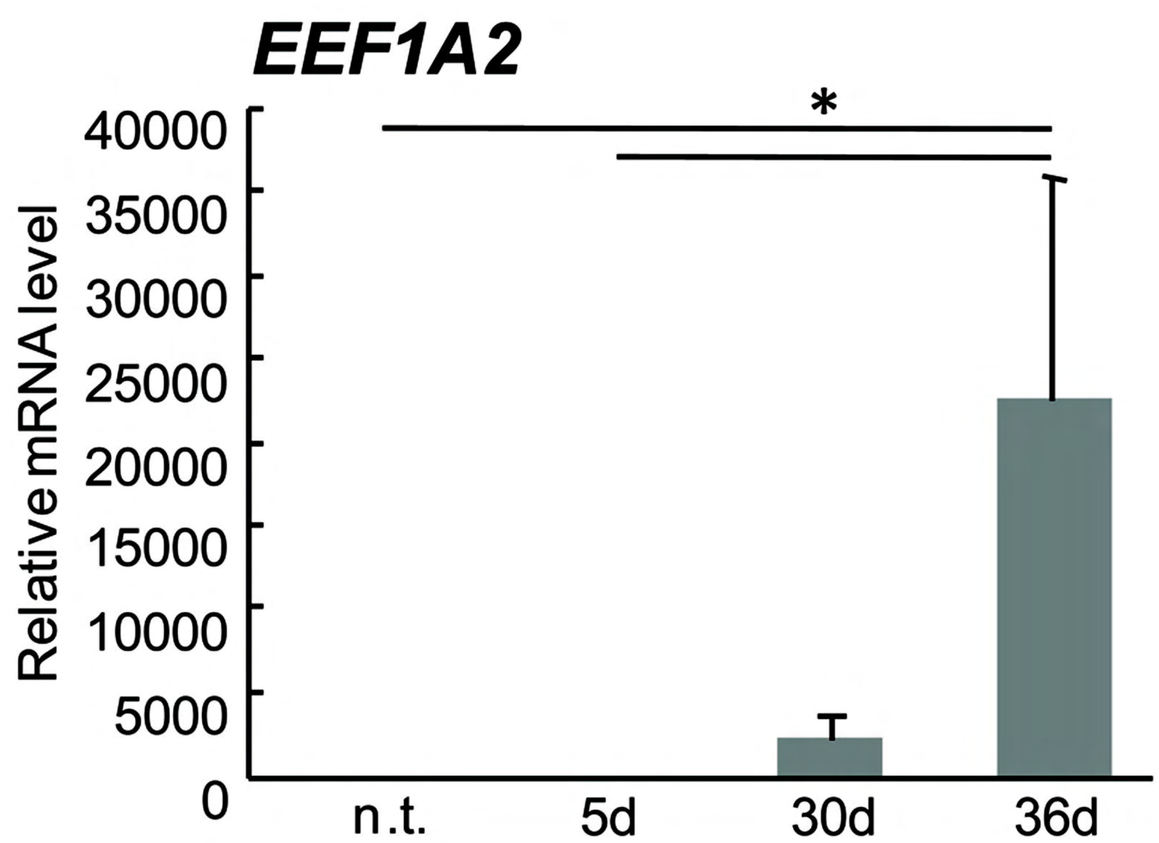
4.0
3.0
2.0
1.0
0.0
*
*
n.t.
5d
30d
36d


EEF1A2
Relative mRNA level
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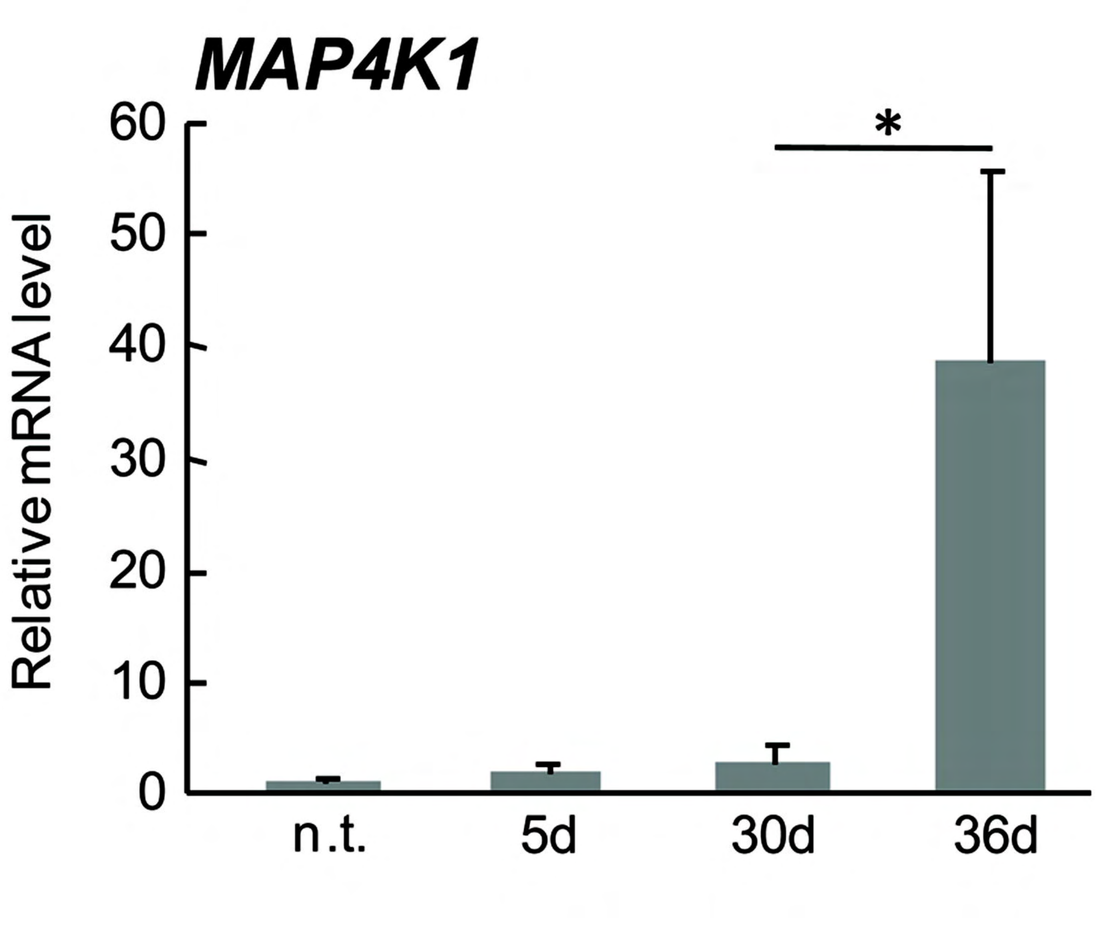
30000
25000
20000
15000
10000
5000
0
*
n.t.
5d
30d
36d


MAP4K1
Relative mRNA level
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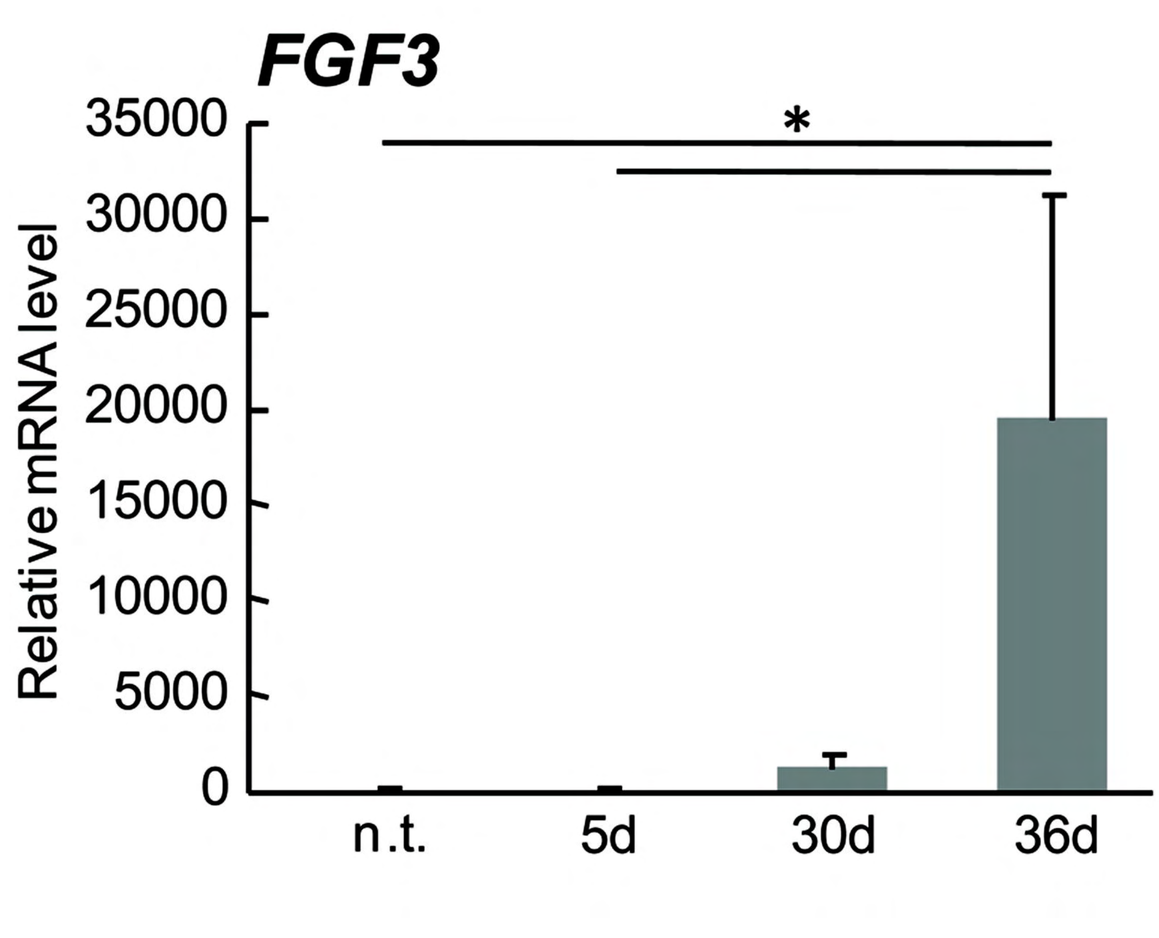
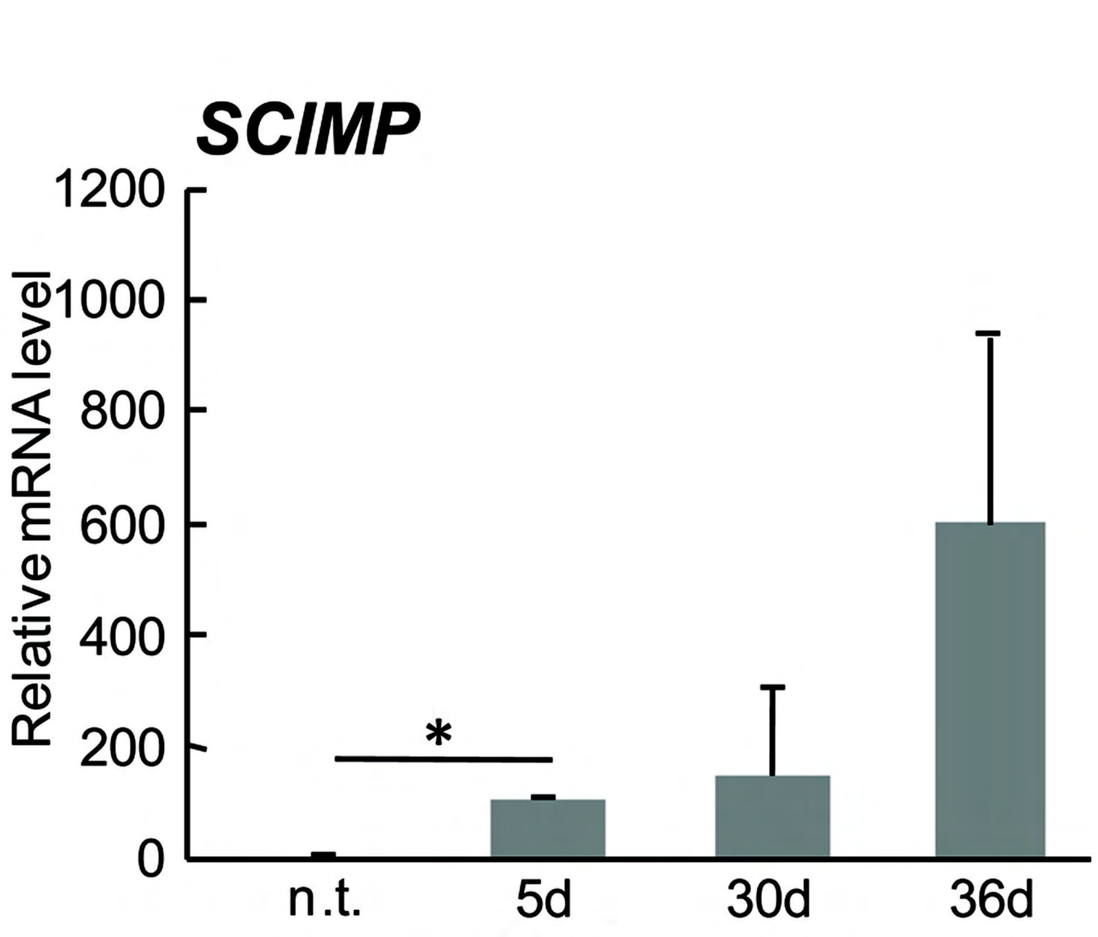
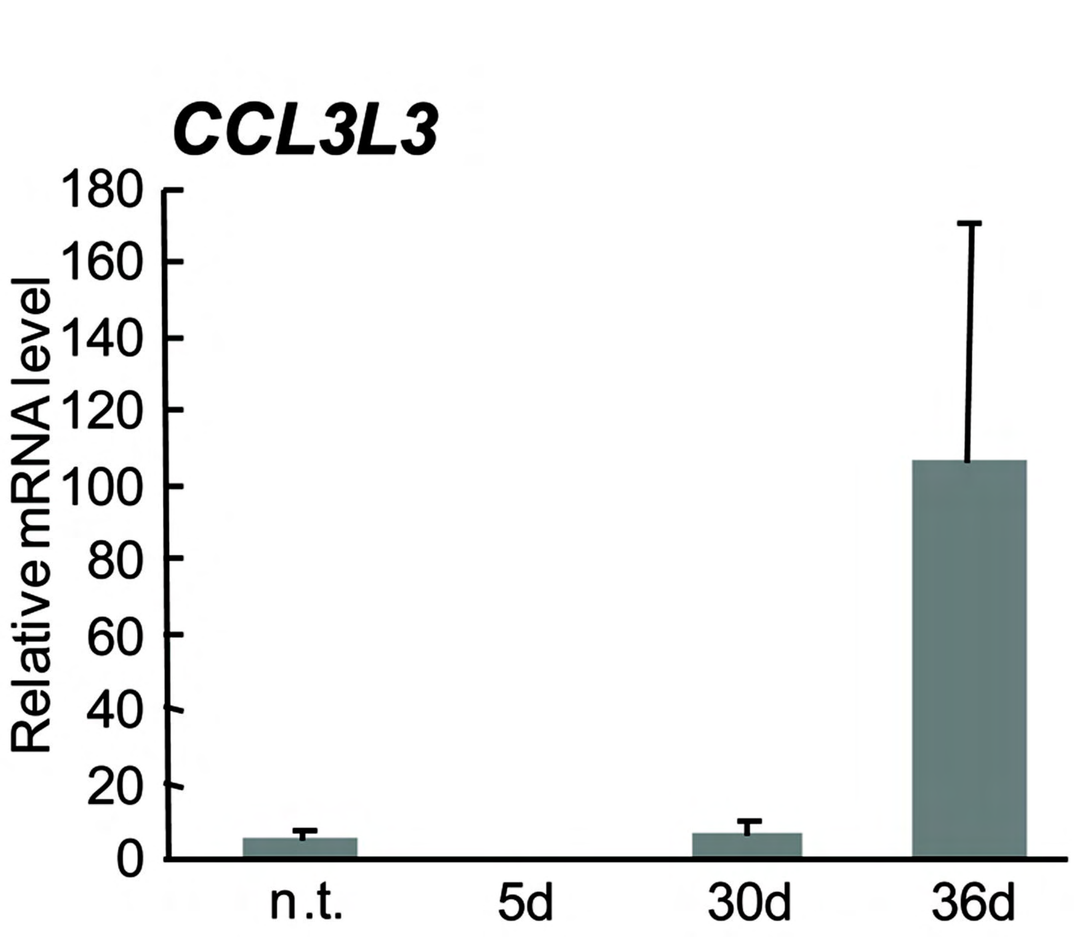
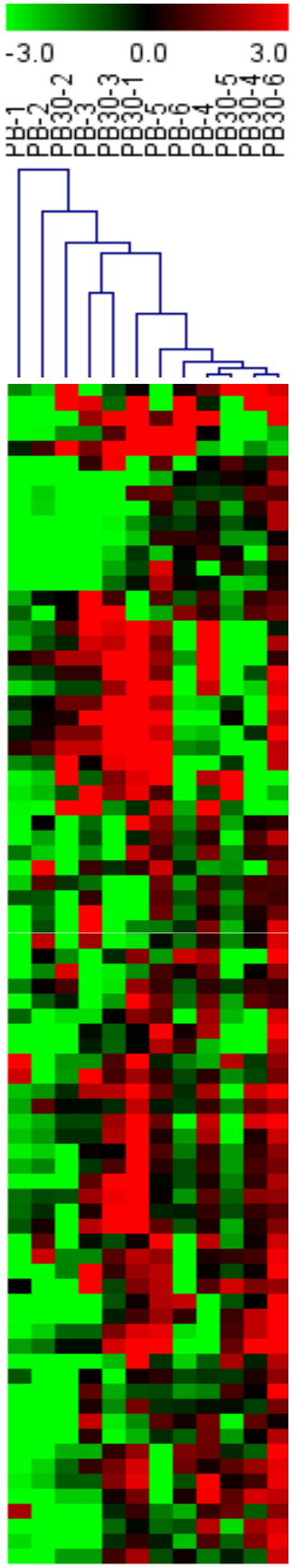
50
40
30
20
10
0
*
n.t.
5d
30d
36d

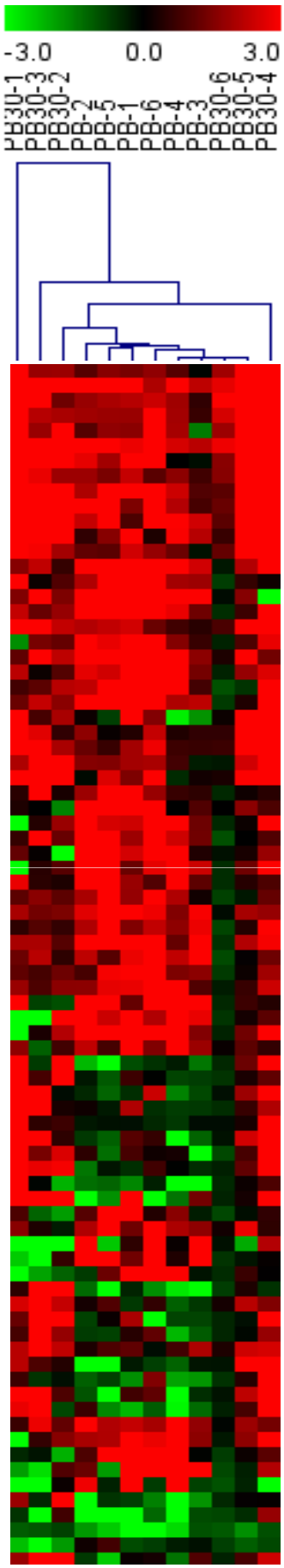

FGF3
Relative mRNA level
35000
30000
25000
20000
15000
10000
5000
0
*
n.t.
5d
30d
36d


SCIMP
Relative mRNA level
1200
1000
800
600
400
200
0
*
n.t.
5d
30d
36d


CCL3L3
Relative mRNA level
180
160
140
120
100
80
60
40
20
0
n.t.
5d
30d
36d



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).