Expanded circulating hematopoietic stem/progenitor cells as novel cell source for the treatment of *TCIRG1* osteopetrosis

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 doi:10.3324/haematol.2019.238261

Received: September 14, 2019.

Accepted: January 9, 2020.

Pre-published: January 16, 2020.

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SUPPLEMENTARY METHODS

Patients and healthy donors. We collected samples of bone marrow (BM) and peripheral blood (PB) from healthy donors for which exceeding materials were available. BM samples from pediatric HD were previously described[1].

Cord blood (CB) cells were purchased from Lonza. Mobilized PB CD34+ cells were positively selected using the RoboSep device (Stemcell Technologies) from peripheral blood of consenting donors of allogeneic stem cells undergoing blood stem cell mobilization.

Vector production. PGK.TCIRG1 lentiviral vector was produced as previously described[2,3], substituting the IDUA transgene with the TCIRG1 cDNA sequence (GeneArt Gene Synthesis, Thermo Fisher Scientific). In the PGK.TCIRG1/dNGFR, we added the dNGFR marker gene, driven by the CMV promoter.

Vector copy number. Genomic DNA was extracted with QIAamp DNA Blood mini kit (QIAGEN), according to manufacturer's instructions. Vector copy number/genome (VCN) was calculated on transduced cells and transduced hematopoietic progenitors as previously described[4].

Expansion. Transplanted day0 equivalents are calculated as ratio between total cells at the end of the expansion and the number of transplanted cells in each NSG mice.

Flow cytometry analysis. Cells were stained with the following anti-human antibodies (BD Pharmingen, Miltenyi Biotec, Biolegend or eBioscience): CD3 clone BW264/56, CD4 clone VIT4, CD8 clone BW135/80, CD13 clone WM15, CD19 clone SJ25C1,

CD34 clones AC136 and 581, CD38 clone HIT2, CD45 clones 5B1 and 2D1, CD45RA clone HI100, CD90 clone 5E10, dNGFR clone ME20.4-1.H4. Viability was determined with live/dead fixable dead cell stain kit (ThermoFisher Scientific). Samples were acquired on FACS Canto II or LSRFortessa (BD) and analyzed with FlowJo software.

The multiparametric whole blood dissection was performed as previously described[1].

RNAseq. Total RNA was used to generate mRNA-Seq libraries using the SMART-Seq v4 Ultra low-input RNA kit for sequencing according to the manufacturer's instructions (Takara Bio). The quality of PCR library products was evaluated with the Bioanalyzer (Agilent). Pooled libraries were sequenced in one lane of a single-end flow-cell on an Illumina HiSeq 3000. On average, 25 million of 50bp single-end reads were generated for each sample.

RNA-seq data were aligned to the human reference genome (GRCh38/hg38) using STAR (v2.7.0d) with standard input parameters. Gene counts were produced with featureCounts (Subread v1.6.3) using GENCODE v29 as reference gene annotation. Read counts were processed using edgeR (v3.20.9), using the standard protocol as reported in the respective manual. Differentially expressed genes were determined considering FDR corrected p-values (FDR <0.05). Gene Set Enrichment Analysis (GSEA) were computed using clusterProfiler (v 3.8.1, http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) on gene lists ranked by logFC considering different datasets (Gene Ontology, KEGG Pathway Database, Reactome Pathway Database, Disease Ontology, Molecular Signatures Database). Heatmaps were produced using pheatmap (v1.0.12) R package.

Data Sharing Statement. Microarray data are available at GEO under accession number GSE132889.

Western Blot. Osteoclasts were lysed with RIPA buffer (ThermoFisher Scientific) and proteins were quantified using Bio-Rad protein assay kit II (Bio-Rad). Proteins were denatured in Laemmli loading buffer and a total of 10-15 μg of proteins were separated by gel electrophoresis in a precast gel SDS-PAGE 7.5% (Mini-PROTEAN® TGX™ Gels, Bio-Rad) followed by blotting onto nitrocellulose membrane. After blocking in 5% skim milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST), the membrane was incubated overnight with mouse monoclonal anti-TCIRG1 (Abnova) or rabbit polyclonal anti-p38 MAPK (Cell Signaling Technology) and appropriate secondary antibody: anti-mouse IgG-HRP or anti-rabbit IgG-HRP (Cell Signaling Technology). Blots were developed using Western Lightning Plus-ECL (Perkin Elmer) and images were acquired using ChemiDoc Imaging System (Bio-Rad).

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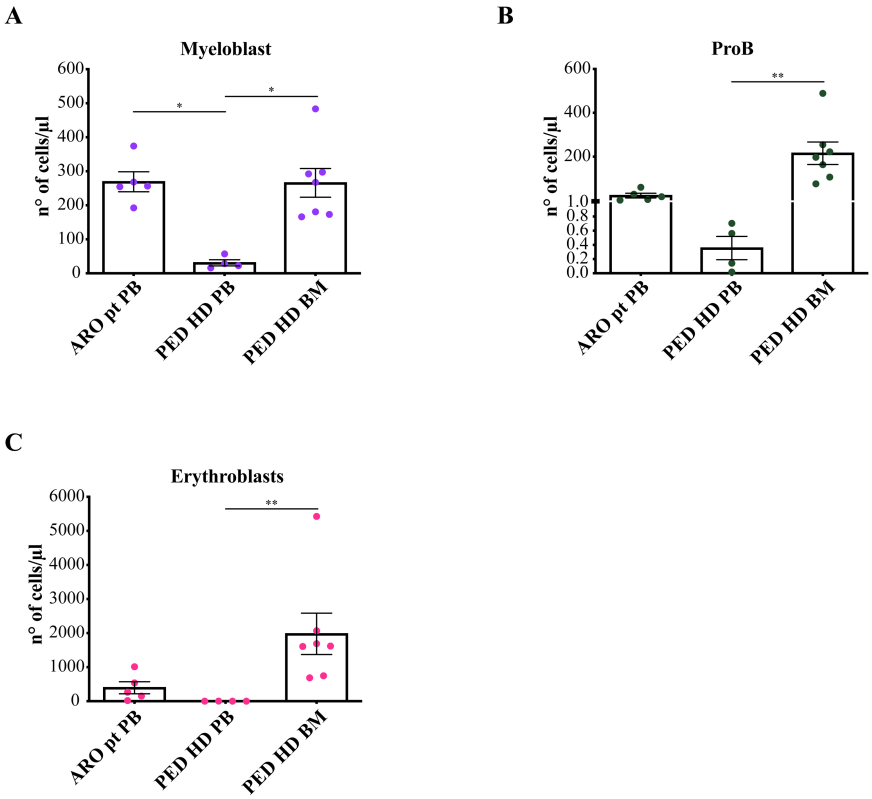
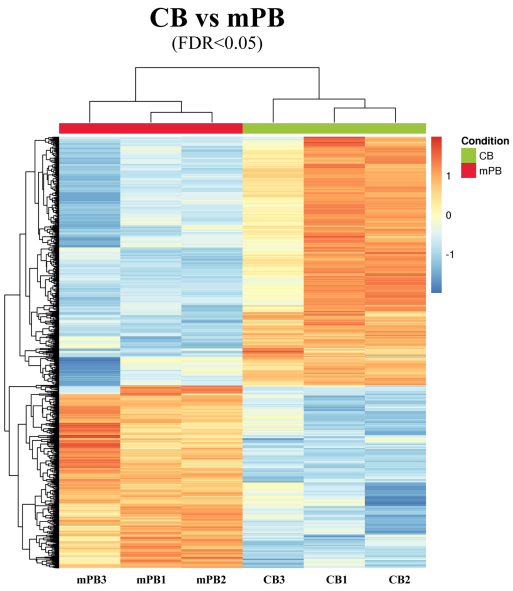
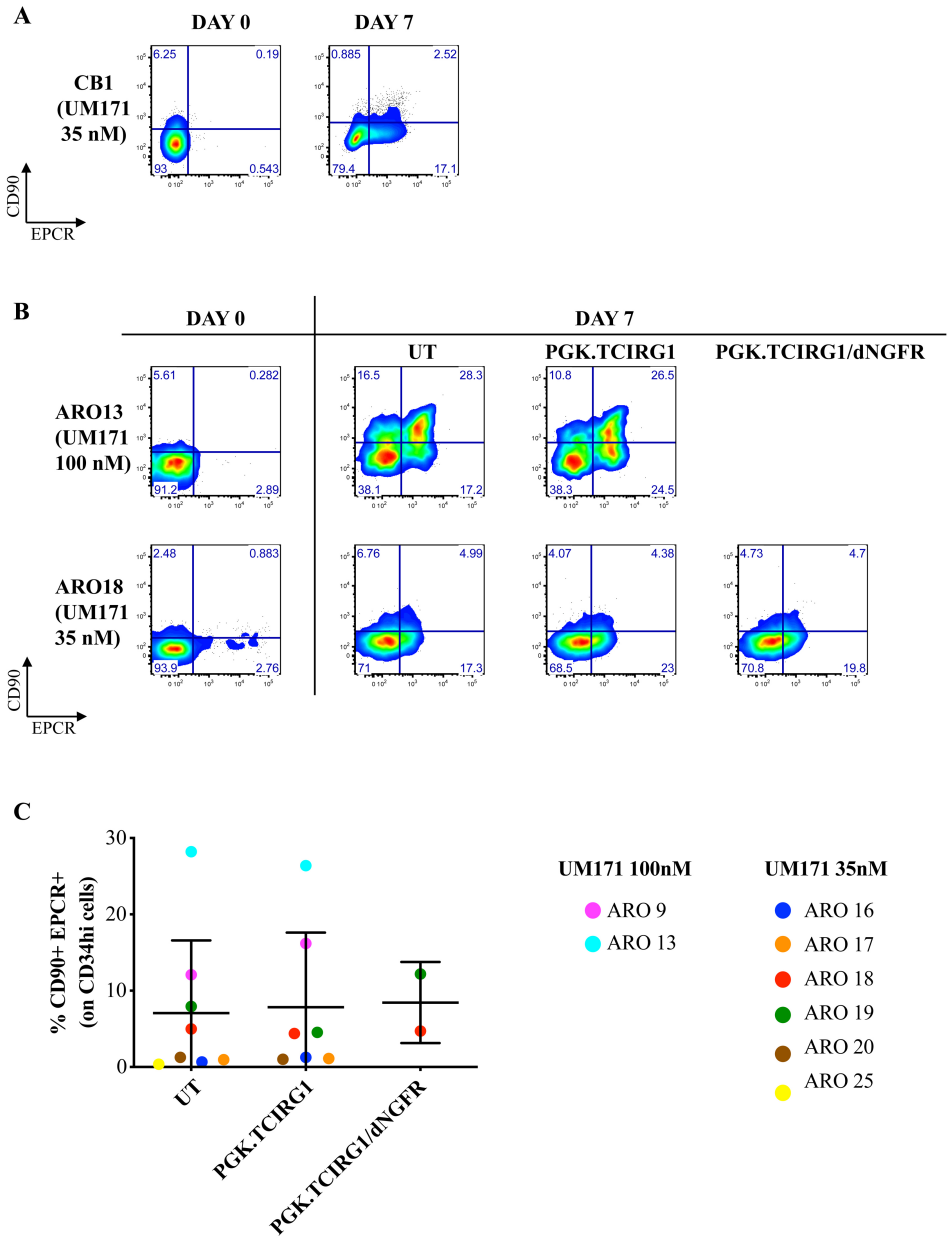
SUPPLEMENTARY FIGURES



Supplementary Figure S1. Immature subsets. Cell count/µl of myeloblasts (**a**), proB (**b**) and erythroblasts (**c**) in ARO patient peripheral blood (n=5), healthy donor peripheral blood (n=4) and bone marrow (n=7). Data show mean ± SEM. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparison post-test.

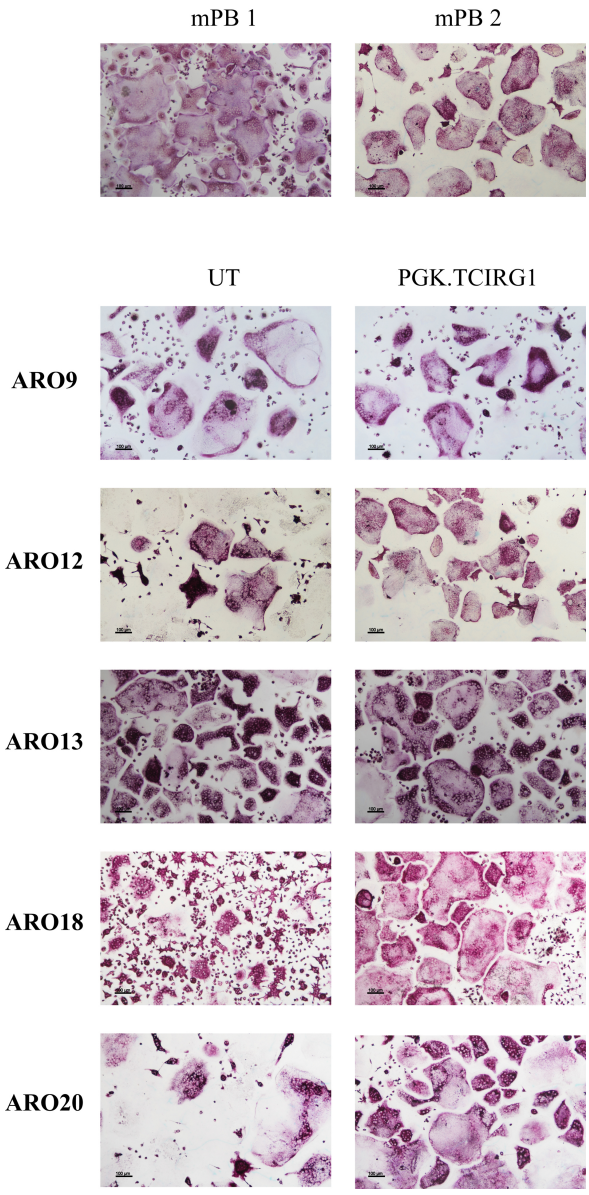


Supplementary Figure S2. RNA sequencing. Heatmap shows unsupervised hierarchical clustering on differentially expressed genes (DEGs) of CB versus mPB.

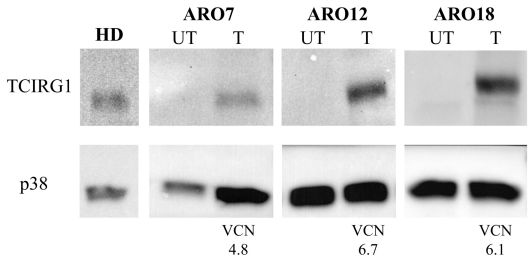


Supplementary Figure S3. HSPC expansion protocol. (**a**) Representative FACS plot of CD34+ cells from a healthy donor cord blood (CB) at day 0 and at day 7. (**b**) Representative FACS plot of CD34+ cells from 2 ARO patients at day 0 and at day 7, cultured with UM171 at a concentration of 35 (ARO18) or 100 nM (ARO13). Plots in panels **a** and **b** show CD90 and EPCR marker expression on the CD34+ population.

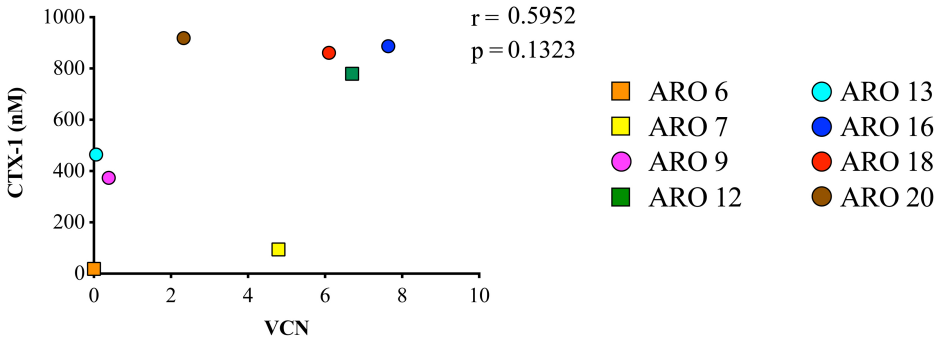
(**c**) Percentage of the $CD90^{+}$ $EPCR^{+}$ population on the $CD34^{high}$ cells at day 7 in untransduced (UT) or transduced (PGK.TCIRG1 and PGK.TCIRG1/dNGFR) patient cells, cultured with 35 or 100 nM UM171, as indicated in figure.



Supplementary Figure S4. TRAP staining. Osteoclasts differentiated on plastic well plates and stained for tartrate-resistant acid phosphatase (TRAP) activity. Images were acquired with Nikon ECLIPSE E600 microscope equipped with Nikon DS-Ri2 camera, using Plan Fluor 4x/0.13 objective and NIS-Elements F 4.30.01 software.



Supplementary Figure S5. Western Blot. Western Blot analysis on total cell lysate of osteoclasts of a healthy donor (HD) and 3 different ARO patients. UT = untransduced, T = transduced with LV.PGK.TCIRG1



Supplementary Figure S6. Correlation VCN/resorption**.** Correlation between vector copy number/genome (VCN) of CD34+ cells and C-terminal telopeptide fragment of type I collagen (CTX-I) release in supernatant of CD34+ derived osteoclast cultures. Statistical significance was determined by Spearman correlation and r value is indicated in figure.

Supplementary Table S1. Primary NSG transplants

Patient	Mouse #	Transplanted with		Transplanted d0 equivalents	% engraftment at 13w			*In vitro* VCN
		Sample	Cell number		Blood	Spleen	Bone marrow	
ARO2	1	ARO2 PGK.TCIRG1/dNGFR	7,00E+05	0.15	34.7	92.9	91.1	0.1
	2		7,00E+05	0.15	33.3	97.0	94.4	
	3		5,00E+05	0.11	11.1	81.5	66.6	
	4	ARO2 UT	5,00E+05	0.11	25.9	92.4	84.8	0.0
	5		7,00E+05	0.15	49.8	97.3	94.9	0.0
ARO7	6	ARO7 PGK.TCIRG1	9,40E+05	0.31	14.7	68.8	65.5	4.8
ARO9	7	ARO9 PGK.TCIRG1	8,00E+05	0.41	1.6	41.6	34.6	0.4
	8	ARO9 UT	5,00E+05	0.26	2.3	40.2	31.3	0.0
ARO13	9	ARO13 UT	6,50E+05	0.39	5.0	45.8	70.8	0.0
	10	ARO13 PGK.TCIRG1	8,00E+05	0.47	7.9	61.0	74.5	0.1
ARO18	11	ARO18 UT	1,00E+06	0.16	0.1	8.0	3.2	0.0
	12	ARO18 PGK.TCIRG1	8,00E+05	0.13	0.9	16.6	13.6	6.1
	13	ARO18 PGK.TCIRG1/dNGFR	1,00E+06	0.16	0.2	6.4	13.0	5.8
ARO25	14	ARO25 PGK.TCIRG1	8,74E+05	0.30	0.8	13.0	23.7	1.4