L-leucine increases translation of *RPS14* and *LARP1* in erythroblasts from del(5q) myelodysplastic syndrome patients

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Supplementary Information

Materials and Methods

Culture of mononuclear cells and differentiation along the erythroid lineage

This study was approved by the local ethics committees (Oxford 06/Q1606/110, Bournemouth 9991/03/E). Bone marrow mononuclear cells from healthy controls were purchased from Lonza. Bone marrow mononuclear cells from del(5q) MDS patients and healthy controls were cultured for 11 days in StemSpan Serum-Free Expansion Medium (SFEM) media (Stemcell Technologies) supplemented with 0.5 U/mL recombinant human erythropoietin (EPO) (Stemcell Technologies), 100 ng/mL stem cell factor (SCF) (Miltenyi Biotec), 10 ng/mL interleukin 3 (IL-3) (Miltenyi Biotec), 40 μg/mL lipids (Sigma- Aldrich), 2 mM L-glutamine (Sigma- Aldrich), and 1% penicillin-streptomycin solution (Sigma Aldrich). Cells were passaged every 2 or 3 days. On day 7, the concentration of EPO was increased to 3 U/ml.[1]

L-leucine treatment

Bone marrow mononuclear cells from del(5q) MDS patients and healthy controls were treated with either L-leucine (Sigma Aldrich) or D-leucine (Sigma Aldrich) at a concentration of 600 μg/ml at day 9 of the culture, as previously described.[2] Culture media containing either L-leucine or D-leucine was refreshed every 2-3 days.

Flow cytometry analysis of erythroid markers

Erythroid differentiation was measured by staining of erythroid cell surface markers CD36, CD71 and CD235a at day 11 of culture. Cells were washed in PBS (Life Technologies) and incubated for 30 minutes on ice with PE anti-human CD36 (cat. 336206, Biolegend) anti-CD71 FITC (cat. 11-0719-42, eBioscience) and anti-CD235a APC (cat. 17-9987-42, eBioscience) antibodies. Cells were washed in PBS (Life Technologies), before staining with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (1μg/ml, Sigma Aldrich). Data acquisition was performed on a BD LSRII instrument (BD Bioscience; Franklin Lakes, NJ, USA) and the data were analyzed using FlowJo software version 10.

Intracellular staining of alpha and beta globin

Mononuclear cells were harvested, washed once in PBS and stained with the Fixable Viability dye eFluor 780 (eBioscience) for 30 min. After PBS washing, cells were fixed and permeabilized using the FOXP3 Fix/Perm Buffer Set (eBioscience), according to the manufacturer's recommendations. The Anti-alpha globin antibody FITC (ab19361, Abcam) was diluted 1/200 and added to the cells. Cells were incubated for 30 minutes in the dark at room temperature. The Hemoglobin β Antibody (37-8) PE (sc-21757, Santa Cruz

Biotechnology) diluted 1/50 was also added to the cells and incubated for 15 min at 4˚C in the dark. Cells were then washed twice in PBS and resuspended in Perm buffer (eBioscience) before flow cytometry analysis.

Sandwich ELISA of RPS6

Total cell lysates were prepared using Cell Lysis Buffer (Cell Signaling) according to manufacturer's instruction. Lysates were vortexed 10 times and placed on ice. 400µl of 1X Cell Lysis Buffer plus 1mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich) was used for 10^7 cells maximum. The amount of protein in each cell extract was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Cell extracts were diluted to 25µg/ml and 100µl of each lysate was added to the appropriate well. Sandwich ELISA was performed to measure the phosphorylation levels of mTORC1 target RPS6 (PathScan Phospho-S6 Ribosomal Protein (Ser235/236)) according to manufacturer's instructions (Cell Signaling Technology). The absorbance (OD450 nm) was measured on a SpectraMax Plus 384 Microplate Reader (Molecular Devices) and it is proportional to the levels of phosphorylated protein in the cell extract.

Polysome profiling

Cycloheximide (Sigma Aldrich) was added to the culture media at a final concentration of 0.1 mg/ml. Cells were incubated in media containing cycloheximide for 10 minutes at 37ºC.[3] Between 200,000 and 1 x 10^6 cells were used in each experiment. Cells were rinsed in 10ml of ice-cold PBS (Life Technologies) containing cycloheximide (Sigma-Aldrich) at 0.1 mg/ml. Cell extracts were prepared according to the TruSeq RiboProfile (Mammalian) Kit protocol (Illumina). 200µl of Mammalian Lysis Buffer was used for 200,000 cells. For each sample, the cell extract was aliquotted into two tubes, one for the isolation of polysome-bound mRNA and a second one for extraction of total mRNA. Complexes of mRNA associated with polysomes were isolated from cell extracts by size-exclusion chromatography using Illustra MicroSpin S400 HR columns (GE Healthcare) as previously described.[3] The columns were vortexed and centrifuged at 735 xg for 1 minute. Between 25µl and 100µl of cell extract was slowly added to the centre of the column, which was then spun at 735 xg for 2 minutes. The purified fraction containing polysome-mRNA complexes was collected at the bottom of the tube. Ribosome-bound mRNA (RBR) was extracted from purified polysome-mRNA fractions using the RNA Clean and Concentrator-5 Kit (Zymo Research). Total RNA was extracted from the remaining cell lysate using the same RNA Clean and Concentrator-5 Kit (Zymo Research), according to the manufacturer's protocol. Total RNA and RBR was treated with DNase I (Invitrogen) according to the manufacturer's protocol prior to either reverse transcription or library preparation for RNA-seq. RNA quality and quantity were assessed by Agilent RNA 6000 Pico Kit (Agilent Technologies) on the Agilent 2100 Bioanalyzer System (Agilent Technologies). Between 10pg and 100pg DNase I-treated RNA was used for RNA sequencing. Library preparation using the Smart-Seq2 protocol[4] was performed at the High-Throughput Genomics Centre (Wellcome Trust Centre for Human Genetics, University of Oxford). Smart-Seq2 library preparation was performed according to the protocol from

Picelli et al (2014)[4] and adaptors were added to cDNA using the Nextera XT DNA sample preparation kit (Illumina). Libraries for total RNA samples and RBR were sequenced on a HiSeq4000 Sequencing System (Illumina) using 75bp paired-end chemistry. Illumina PhiX Control was added at approximately 1%. Approximately 50,000,000 reads per sample were obtained.

RT-qPCR

For use in RT-qPCR assays, DNAse I-treated RNA was reverse transcribed prior to amplification. A maximum of 1µg RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. Reverse transcription was performed using random hexamers. Taqman RT-qPCR amplifications reactions were prepared using LightCycler 480 Probes Master Mix (Roche) and the following TaqMan assays (Applied Biosystems): Hs00735285_m1 (*RPS14*), Hs00828752_gH (*RPS20*), Hs01375212_g1 (*RPS18*). The *β2-microglobulin* gene (*B2M*) *(*Hs00430330_m1, Applied Biosystems) was used to normalize for differences in input cDNA and gene expression was calculated using the ΔΔCT method for relative quantification.

Analysis of Gene expression

In order to study gene expression changes in MDS with del(5q), total RNA fractions of patient-derived erythroblasts and erythroblasts from healthy controls treated with D-leucine were analyzed. The software package Kallisto[5] was used to estimate transcript abundance as transcripts per million (TPM). TPM values for 18361 genes were analyzed using Gene Set Association Analysis for RNA-Seq with Sample Permutation (GSAASeqSP).

Analysis of Polysome profiling data

Quality control of sequencing data was performed using RNAseQC[6] on *fastq* files to analyze read metrics (such as read length, mapping and duplicate reads) and coverage (mean coverage and 5'/3' bias). Sequencing reads were mapped using the software STAR[7] and *bigwig* files were made in order to visualise each mapped BAM file using the UCSC Genome Brower (https://genome.ucsc.edu/) and verify the absence of DNA contamination. Custom scripts were written for the analysis of sequencing data for gene expression and polysome profiling. Read counts per gene were calculated using featureCounts and normalized using the software DEseq2 (version 1.14.1)[8] for RBR and total RNA from erythroblasts derived from patients with del(5q) MDS and from healthy controls treated with D-leucine. The translation efficiency (TE) of each transcript in each sample was calculated as the ratio between the read count per transcript in the RBR fraction of one sample and the read count for the same transcript in the total RNA fraction of the same sample. The Fold change (FC) in TE of each gene was calculated as the ratio between the TE for that gene in L-leucine treated samples and the TE in D-leucine treated samples or in patients samples over samples from healthy controls. The $\log_2$ of the average FC for each gene was calculated and plotted.

GO analysis

The Compute Overlaps tool of the molecular signatures database (MSigDB, Broad Institute) was used to evaluate the overlap of mRNAs significantly suppressed in del(5q) MDS patient-derived erythroblasts with gene ontology (GO) gene sets in the MSigDB collection.

Python script for Analysis of 5' TOP and 5'TOP-like motifs

MRNAs were classified into 5'TOP and 5' TOP-like using a customised Python script (https://github.com/jbkerry/NGS/blob/master/5top_mrna.py). The script analyses sequences immediately downstream of the transcription start site (TSS) following the definition of 5'TOP and 5' TOP-like mRNAs by Thoreen *et al.* (2012).[9] The TSS used in the analysis were obtained from the UCSC database (hg19, https://genome.ucsc.edu/).

Statistical analysis

Flow cytometry data for expression of erythroid cells surface markers and intracellular staining of alpha and beta globin were analyzed using a two-way ANOVA to compare the difference in protein phosphorylation between 5q- patients and healthy controls and between L-leucine and D-leucine treated samples. Two-way ANOVA was also used for the analysis of data from ELISA experiments. A p value <0.05 was considered significant.

Statistical analysis was performed using custom R scripts (version 3.3.1). A z score for each mRNA was calculated by dividing the difference between the TE for one gene and the mean TE of all genes, by the standard deviation. Genes with z scores lower than -1.5 or higher than 1.5 were considered differentially translated.

A two-tailed Mann–Whitney U test was used to analyze the difference in TE of all genes between 5q- patients and healthy controls and between L-leucine and D-leucine treated samples. A p value <0.05 was considered significant.

Supplementary references for Materials and methods

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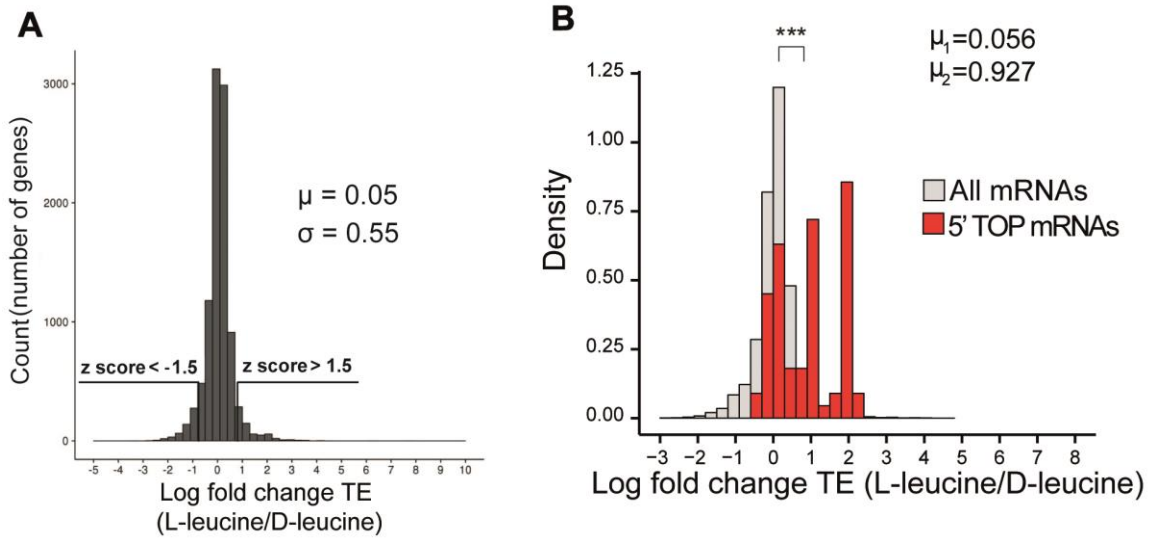
Supplementary Table 1. Subtype and karyotype of del(5q) MDS patients analyzed.

Patient ID	Subtype	Karyotype
MDS1	RA (5q- syndrome)	46,XX,del(5)(q13q33) [19] / 46,XX [1]
MDS2	RA (5q- syndrome)	46,XX,del(5)(q22q35) [19] / 46,XX [1]
MDS3	RAEB	46,XX,del(5)(q13q33) [20]
MDS4	RA (5q- syndrome)	46,XX,del(5)(q13q33) [16]/ 46,XX [6]
MDS5	MDS del(5q)	46,XX,del(5)(q13q33) [13] / 46,XX [6], an additional mitosis carries trisomy 8
MDS6	RA (5q- syndrome)	46,XX,del(5)(q13q33) [6] / 46,XX [14]
MDS7	RA (5q- syndrome)	46,XY,del(5)(q13q33) [20]
MDS8	MDS del(5q)	46,XY,del(5)(q?)

Supplementary Figure 1. A. Histograms showing the log2 fold-change in TE of every mRNA in L-leucine compared to D-leucine-treated healthy controls. The y-axis represents the number of genes for a given log2 fold-change values. Log2 FC between -10 and 10 are shown. Extreme log2FC values were not shown for visualization purposes. Transcripts with a Z score <-1.5 (corresponding to a LogFC of -0.77) or > 1.5 (corresponding to a LogFC of 0.89) were considered differentially translated. μ is the mean and σ the standard deviation. **B.** Histogram showing the log2 fold-change in TE of 83 known 5'TOP and 5'TOP-like genes (red) in D-leucine- over L-leucine-treated erythroblasts from healthy controls and the TE of all mRNAs measured (grey). A Mann-Whitney U test was performed. μ_1 is the mean of the logFC in TE of all mRNAs while μ_2 is the mean logFC in TE of 5'TOP mRNAs. *** = p<0.001.



Supplementary Figure 2. Normalized levels of phosphorylated S6K1 measured by sandwich ELISA in healthy controls and del(5q) MDS patients treated with D-Leucine (white) or L-leucine (orange). * = $p<0.05$; ns = not significant. Error bars represent the SEM of three biological replicates.