SUPPLEMENTARY APPENDIX

In vivo activation of the human δ-globin gene: the therapeutic potential in β-thalassemic mice

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Supplemental Methods

Constructs

CACCCδ-LCR construct has been obtained by cloning a 4.7 Kb KpnI fragment containing the δ-globin gene driven by the wt or CACCC containing δ-globin gene promoter (CACCCδ-LCR) into the GSE 1417 vector.[16]

Generation of transgenic mice

DNA fragments were linearized by restriction enzyme digestion (SacII), purified by Elutip-D syringe column (Whatman). Transgenic mice were generated by microinjection of the purified DNA into the pronuclei of the fertilized eggs of FVB/N mice and then transferred into the oviduct of pseudopregnant foster mothers from a mixed strain (C57/BL10xCBA/J). Genotype was determined by PCR using the following primers: δ promoter Fw, 5'-GAGGCAAAGAAGAACTT-3'; δ promoter Rev, 5'-GTCTGTTTGAGGTTGCT-3'; as well as Southern Blot analysis.

Copy number analysis.

Real-time quantitative PCR (qPCR). analysis was performed to accurately calculate the δ-globin gene copy number. Real-time qPCR primers for human δ-globin gene (target gene) were as follows: Fw, 5'-TGAAACCCTGCTTATCTTAAACCAA-3'; Rev, 5'-TTATGTCAGAAGAAAGTGTAAGCAACAG-3'; primer sequences for mouse εy gene (reference gene) were the following: Fw, 5'-CCTCTGCTTCTGCCATAATGG-3'; Rev, 5'-CCAAAAGCAGTCAGCACCTTCT-3'. All the values obtained were normalized using a transgenic mice line (Line 72)[17] carrying a single copy of the full human β-globin locus as a control.

RNA extraction and retrotranscription

Total RNA was extracted using the standard TRIzol method according to the manufacturer's instructions (Invitrogen). RNA concentrations and purity were measured using a NanoDrop 2000 C Spectrophotometer (Thermo Scientific) and quality was verified by agarose gel electrophoresis.
First-strand cDNA was generated by reverse transcription of 1 μg total RNA per sample with random hexamer using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions in a final reaction volume of 20 μl.

Gene expression analysis

Gene expression was assessed by S1 protection assay, carried out as in Stroubulis J et al. 1992[17] and Poddie et al. 2000.[18] Quantification of the signal obtained from the S1 analysis was obtained by using a Phosphorimager Molecular Dynamic (Amersham Biosciences, UK).

RT-qPCR was performed to measure the δ-globin gene mRNA expression by using the following PCR primers and probes: δ-globin gene (Hs00426283_m1) as target gene, eukaryotic 18S rRNA (cod. 4319413E) and GAPDH (cod. 4308313) as reference genes, and TaqMan Universal PCR Master Mix (all from Applied Biosystems).
Assays were run on a 7900 HT Fast Real Time PCR System (Applied Biosystems) using universal cycling conditions. Quantification of copy numbers and gene expression was performed using the comparative C(t) method.[35]

Hematological analysis

Blood samples from wild type, Hbbth3/+ and Hbbth3/+CACCCδ-LCR (homozygous and heterozygous) adult mice were obtained by retro-orbital puncture under anesthesia. Total hemoglobin levels, red cell counts, hematocrit levels, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW) were determined by using an Automated Hematology Cell Counters MS4 (Melet Schloesing Lab) and the HemoCue Hemoglobin System.

Flow cytometry

For fluorescence-activated cell sorting (FACS) analysis, $1x10^6$ bone marrow or spleen cells were washed and incubated on ice for 15 minutes with 0.1 μg of FITC-labeled anti–mouse CD71 and PE-conjugated anti-mouse Ter119 antibodies (Biolegend San Diego CA) in PBS-1% BSA. Control samples were incubated with 0.1 μg FITC-labeled anti–mouse IgG_1 and PE-conjugated rat IgG_{2b} isotype control antibodies (Biolegend San Diego CA). Results were analyzed using Flow-Jo software (Tree Star, Ashland, OR).

Supplemental Data

Ratio between early precursor ($Ter119^+/CD71^+$) and more mature erythroid cells ($Ter119^+/CD71^-$)

In the spleen and BM, the amount of IE was estimated based on the ratio between early precursor ($Ter119^+/CD71^+$) and more mature erythroid cells ($Ter119^+/CD71^-$). On average, the ratios between $Ter119^+/CD71^+$ and $Ter119^+/CD71^-$ cells in the spleen were 0.36 ± 0.1; 1.46 ± 0.1; 1.97 ± 0.2 and 4.9 ± 2 respectively, in wt, Hbbth3/+ CACCCδ-LCR homozygous, heterozygous and Hbbth3/+ mice at 2.5 months of age. In the bone marrow cells population ratios were 2.4 ± 0.8; 3.5 ± 0.5; 6.5 ± 1.3 and 8.5 ± 1.8 respectively, in wt, Hbbth3/+CACCCδ-LCR homozygous, heterozygous and Hbbth3/+ mice at 2.5 months.

Spleen weight

In Hbbth3/+ CACCCδ-LCR homozygous mice the spleen weight, normalized to the body weight, was 9,33± 1.3 (t-test p=3.1E-05); In Hbbth3/+ CACCCδ-LCR heterozygous mice was 13.8 ± 2.2 (t-test p=0.048); in Hbbth3/+ was 16.9 ± 1.9; in wt mice was 3 ± 1.1.

Iron contents

In the spleen the iron content were 11.06 ± 2.3; 252.19 ± 43.2 (t-test p=0.0012); 436.76 ± 34.3 and 374.44 ± 29.7 respectively, in wt, Hbbth3/+ CACCCδ-LCR homozygous, heterozygous and Hbbth3/+ mice at 2.5 months of age. In the liver were 86 ± 14; 152.33 ± 19.1 (t-test p=0.000104); 246.42 ± 27.5 and 248.61 ± 21.3 respectively, in wt, Hbbth3/+CACCCδ-LCR homozygous, heterozygous and Hbbth3/+ mice. In the heart were 5.02 ± 0.67 (t-test p=0.00069); 5.01 ± 0.3 (t-test p=0.006); 6.69 ± 0.5 and 10.27 ± 2 respectively, in wt, Hbbth3/+CACCCδ-LCR homozygous, heterozygous and Hbbth3/+ mice. All the value were measured at 2.5 months and were normalized to the organ weight.

Notably however, at 5 months also the Hbbth3/+CACCCδ-LCR heterozygous mice show a significant decrease of iron content in spleen and in the liver. In the spleen the iron content were: 592.84± 8.61 (t-test p=2.73E-05) compared to the Hbbth3/+ mice: 877.17 ± 2.7, while the iron content in the wt mice were 36.45 ± 23.7. In the liver the iron content were 108.34 ± 62.3; 207.19 ± 48.3 (t-test p=0.0037); 409.61 ± 83.6 respectively, in wt, Hbbth3/+CACCCδ-LCR heterozygous and Hbbth3/+ mice.

Supplemental bibliography

35) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25:402-408.