

Material and Methods

In Vitro Expansion of Erythroid Progenitors:

We adapted the mouse erythroid progenitor expansion assay (1) (2) to develop a human erythroid expansion protocol. We isolated peripheral blood mononuclear cells (PB-MNCs) on a gradient of Ficoll-Hypaque and expanded the erythroid progenitors in 3 week liquid expansion protocol with a typical yield of $\sim 2 \times 10^6$ cells from 7 ml of anticoagulated blood. The cells were harvested at day 18, which corresponds to late normoblast differentiation as determined by flow cytometric analysis using anti-human FITC-conjugated CD71 (transferrin receptor) monoclonal and phycoerythrin (PE) conjugated anti-human glycophorin A (GPA) (BD Pharmingen, San Diego, CA) antibodies and morphologic analysis (3).

Analysis of splice variants:

Total RNA was isolated and cDNA synthesized as described(3). β -beta globin cDNA from the reticulocytes and expanded erythroid progenitors were amplified (PTC-200, MJ Research, USA) using two different primer sets: a) First set (proximal exon 1 and distal exon 2): sense primer, 5'-GAGAAGTCT GCCGTTACTGC-3', antisense primer 5'-AGCCAGGCCATCACTAAAGG-3'; b) Second set (5' and 3' untranslated regions): sense primer, 5'-CTGTGTTCACTAGCAACCTC-3', antisense primer 5'-GGACAGCAAGAAAGCGAGC-3'.

β -beta globin DNA and cDNA sequencing:

β -beta globin DNA and cDNA sequencing was performed by ABI3730 96-capillary sequencer.

Haplotype analysis:

Genomic DNA was isolated and β -beta globin haplotype analysis performed utilizing five restriction sites: Hind III site at G γ -gamma gene, Hinc II site 5' to ψ -Psi β -beta gene, Hinc II site 3' to ψ -Psi β -beta gene, Ava II site at β -beta-gene, and Bam H1 site 3' to β -beta-gene as described (4).

Real-Time Quantitative PCR Assay for β -beta globin gene expression:

Quantitative polymerase chain reaction (qRT-PCR) was carried out in an ABI 7000 HT Sequence Detection System using TaqMan master mix and the protocol of the manufacturer (Applied Biosystems) as described previously (3). The reaction was initiated at 50° C for 2 minutes and 95° C for 10 minutes followed by 40 cycles of denaturation at 95° C for 15 seconds and annealing/extension at 60° C for 1 minute. Data were recorded as cycle threshold (Ct) using analytic software available from the manufacturer. The primers and probe for human β -beta-globin gene were used as described (5). All data were normalized using the endogenous human 18S RNA control using primers and probes for human 18S RNA from the Applied Biosystem database. Standard curves were obtained by serial dilution. The amplification efficiencies of the target and control genes were comparable. All reactions were run in triplicates and Ct values were averaged. The target amount was then divided by the 18SRNA amount to obtain a normalized value. β -beta globin gene expression (18S RNA normalized) values from three healthy controls were averaged and designated as the calibrator and given a relative value of 1.0. The quantity of β -beta globin gene expression (18S RNA normalized) in our subjects were compared to the average

value from three healthy controls. All quantities of β -beta globin gene expression in our subjects were expressed as percentage relative to the calibrator.

References:

1. Karur, V, Lowell, CA, Besmer, P, Agosti, V, Wojchowski, DM. Lyn kinase promotes erythroblast expansion and late-stage development. *Blood*. 2006 108:1524-32.
2. Zhang, J, Socolovsky, M, Gross, AW, Harvey, F, Lodish, HF. Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. *Blood* 2003 102 3938-46.
3. Jiang, J, Best, S, Menzel, S, Silver, N, Lai, MI, Surdulescu, GL, Spector, TD, Thein, SL. cMYB is involved in the regulation of fetal hemoglobin production in adults. *Blood*. 2006;108:1077-83
4. Venkatesan, R, Sarkar, R, Old, JM. beta-Thalassaemia mutations and their linkage to beta-haplotypes in Tamil Nadu in southern India. *Clin Genet*. 1992;42:251-6.
5. Irenge, LM, Robert, A, Gala JL. Quantitative assessment of human beta-globin gene expression in vitro by TaqMan real-time reverse transcription-PCR: comparison with competitive reverse transcription-PCR and application to mutations or deletions in noncoding regions. *Clin Chem*. 2005;51:2395-6.