NRF2 mediates γ-globin gene regulation and fetal hemoglobin induction in human erythroid progenitors

Xingguo Zhu,[1] Biaoru Li[1] and Betty S. Pace[1,2]

[1]Department of Pediatrics, Division of Hematology/Oncology and [2]Department of Biochemistry and Molecular Biology, Augusta University, Augusta, GA, USA

Correspondence: xzhu@augusta.edu
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Supplementary Appendix

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Xingguo Zhu,[1,*] Biaoru Li,[1] Betty S. Pace[1,2]

[1]Department of Pediatrics, Division of Hematology/Oncology; [2]Department of Biochemistry and Molecular Biology, Augusta University, Augusta, GA 30912

*Corresponding Author: Xingguo Zhu, PhD, Department of Pediatrics, Division of Hematology/Oncology, Augusta University, 1120 15th St., CN-4116, Augusta GA 30912

Email: xzhu@augusta.edu

Phone 706-721-6895

Fax 706-721-7271

Supplemental Methods

Methods

Tissue culture and reagents

Human bone marrow $CD34^+$ stem cells (ReachBio, Seattle, WA) were cultured in a modified two-phase liquid culture system.[1] During phase 1, cells were grown in Iscove's Modified Dulbecco medium (IMDM) with 15% fetal bovine serum, 15% human AB serum, 10 ng/mL interleukin-3, 50 ng/mL stem cell factor and 2 IU/mL erythropoietin (Sigma-Aldrich, St. Louis, MO). Phase 2 was initiated on day 7 with a similar medium, except the growth factors stem cell factor and interleukin-3 were removed. Giemsa staining was used to monitor cell morphology; Dimethyl fumarate (DMF) purchased from Sigma-Aldrich (St. Louis, MO) was dissolved in IMDM and used at 200 μM on day 8 for 48 hours. Cells were harvested for cell count and viability using 0.4% Trypan blue exclusion. KU812 cells were cultured in IMDM with 10% fetal bovine serum as previously published by our lab.[2]

Lentiviral shRNA particle transduction

NRF2 or small MAF lentiviral particles (shNRF2 and shMAF) were produced by standard methods employing co-transfection of pLKO.1 shRNA and packaging vectors in HEK 293FT cells (Invitrogen, Carlsbad, CA). The shNRF2 constructs targeted the human NRF2 mRNA sequence 5'-guggcugcucagaauugcaga-3' (NM_006164). The shMAF construct targeted the human MAFF mRNA sequence 5'-cuggagcucgacgcgcugcgc-3' (NM_001161572). A standard scramble sequence (shScr) against the nonspecific sequence 5'-ccuaagguuaagucgcccucg-3' was used as a control. To establish primary erythroid progenitor stable lines cells were transduced with shNRF2 or shMAF viral particles on day 4; after 48 hours 0.8 μg/mL puromycin (Invitrogen, Carlsbad, CA) was added until analysis on day 10. For KU812 stable cell lines, shNRF2 and shMAF lentiviral particles were transduced as described above and selected with puromycin (1.5 μg/ml) until analysis.

RNA isolation and RT-qPCR analysis

Total RNA was isolated and analyzed as previously published.[2] To quantify mRNA levels for ε-, γ- and β-globin and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH), gene-specific primers were used (Supplementary Table S1). To quantify NRF2, NQO1, CD71 and CD235a, we used the RT2-qPCR Primer system (Qiagen, Germantown, MD). All mRNA levels were normalized to GAPDH before analysis.

Western blot and immunoprecipitation

Western blot analysis was performed as previously published.[2] Antibodies against HbF (51-7), HbA (37-8), NRF2 (H-300), MAF (D-12), and TBP (N-12) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibody against NQO1 (ab80588) was purchased from Abcam (Cambridge, MA) and antibodies against β-actin (A5316) and rabbit normal IgG (I8140) were purchased from Sigma (St. Louis, MO). For co-immunoprecipitation experiments, cell lysates were pre-cleared with protein A-agarose and supernatants were immunoprecipitated with anti-NRF2 antibody followed by western blot with anti-MAF antibody.

Immunofluorescent microscopy

Immunofluorescence studies were performed with anti-NRF2 and anti-MAF antibody followed by secondary Fluorescein isothiocyanate (FITC) labeled anti-rabbit antibody and Phycoerythrin (PE) labeled anti-mouse antibody (Invitrogen, Carlsbad, CA). The stained cells were mounted with fluorescent medium (Vector Laboratories, Burlingame, CA) and microscopy was performed using a Zeiss Axio Imager M1; cell images were acquired using Axiovision software.

Flow cytometry analysis

To measure the HbF positive cells (%F-cells), erythroid progenitors were fixed with 1% formaldehyde and stained with FITC anti-HbF antibody (ab19365, Abcam); isotype control IgG antibody (MBS524511, MyBioSource, San Diego, CA) was used to detect non-specific staining.

Erythroid progenitor differentiation was determination by FITC-CD71 and Allophycocyanin (APC) labeled CD235a antibodies staining. The %F-cells levels and CD71 and CD235a expression were analyzed on a LSR-II flow cytometer (BD Biosciences, San Jose, CA).

ChIP and sequential-ChIP assay

ChIP and sequential-ChIP were performed as previously reported[3] with NRF2 (H-300), MAF (D-12) and TATA binding protein (TBP; N-12) antibodies (Santa Cruz Biotechnology). The primers used to quantify *in vivo* DNA binding to the regions are summarized in Supplementary Table S1. The Gγ-globin cAMP response element (G-CRE) was used as a negative control region. Rabbit IgG was used as ChIP pulldown control.

Chromosome conformation capture (3C) assay

The 3C assay was performed as previously described using *Eco*RI restriction digestion of the *HBB* locus.[3,4] The relative crosslinking frequency between different gene regions was quantified by qPCR using published primers (Supplementary Table S1).[4,5] To adjust for differences in the crosslinking frequency and ligation efficiency between samples, the PCR reactions were normalized to *KTM2B*, a constitutively expressed histone modification gene. An additional control was added to adjust for PCR amplification efficiency using a 179-Kb BAC clone (CTD-3055E11) (Invitrogen, Carlsbad, CA) containing the human *HBB* locus digested with *Eco*RI, self-ligated and used as control templates.

Statistical analysis

Data from at least 3-5 independent experiments were reported as the mean ± standard error of the mean (SEM). The Student's t-test was performed to determine significance and $p<0.05$ was considered statistically significant.

References

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Supplementary Table S1. Summary of primer sequences.	
RT-PCR primers 5'→3'	
GAPDH forward	GAAGGTGAAGGTCGGAGT
GAPDH reverse	GAAGATGGTGATGGGATTTC
ε-globin forward	GTGGATCCTGAGAACTTCA
ε-globin reverse	GCTTTCTCTCAAGGCCAAGCCCAGT
γ-globin forward	GGCAACCTGTCCTCTGCCTC
γ-globin reverse	GAAATGGATTGCCAAAACGG
β-globin forward	CTCATGGCAAGAAAGTGCTCG
β-globin reverse	AATTCTTTGCCAAAGTGATGGG
ChIP assay primers 5'→3'	
HS2 ARE forward	CCTTCTGGCTCAAGCACAGC
HS2 ARE reverse	ATAGGAGTCATCACTCTAGGC
γ-globin ARE forward	CTGAAACGGTCCCTGGCTA
γ-globin ARE reverse	CTGTGAAATGACCCATGGCG
γ-globin G-CRE forward	AAATCGAGGAATAAGACAGTTATGG
γ-globin G-CRE reverse	ACAAGTGAATCTTAATTGCTCCTC
NQO1 ARE forward	ATTACCTGCCTTGAGGAGCA
NQO1 ARE reverse	CAGAGGCCTCAAAAATCTGG
3C assay primers 5'→3'	
HS432	CCAAATGGGTGACTGTAGGGTTGAGA
HS1,	CACAGTTTCTTTAAGGAGG
ε-globin	ATTAACCAATGGTATCTTTCTGAGCA
ε/Gγ intergenic region	CCACCCCGATAAAGATTTTTCTCCATCA
Gγ-globin	GGGTTCATCTTTATTGTCTCCT
BGLT3	TTGCCATACCTCATATCCTTAG
β-globin	GCTCGGCACATGTCCCATCCAG
3' β-globin	AGCTTAGTGATACTTGTGGGCCA
3' HS1	ATTCCCGTTTTTATGAAATCAACTTT
KTM2B primer 1	ACTGCCAGAACCTGAGGAGCAG
KTM2B primer 2	GGGACGGCTCTTCCCCATTGGCT
BGLT3, β-globin locus transcript 3; KTM2B, Lysine Methyltransferase 2B	

Supplementary Table S2. HBB-locus antioxidant response elements (ARE) motifs predicted by JASPAR *in silico* analysis (http://jaspar.genereg.net/).

Start*	End*	Strand	Sequence 5'→3'	Annotation
5,309,730	5,309,720	-1	ATGACTTTGCC	100bp 5' of HS4 core
5,306,148	5,306,138	-1	TTGACTCAGCA	HS3 core
5,306,120	5,306,110	-1	GTGACTTTGCG	HS3 core
5,301,992	5,302,002	+1	ATGACTCAGCA	HS2 core
5,286,552	5,286,562	+1	TTGACAAAGCA	4.5Kb 3' of ε-globin start codon
5,286,028	5,286,038	+1	ATGACCAAGCT	5Kb 3' of ε-globin start codon
5,284,080	5,284,090	+1	TTGACCCAGCA	7Kb 3' of ε-globin start codon
5,280,011	5,280,021	+1	ATGACACAGCA	4Kb 5' of Gγ-globin start codon
5,279,571	5,279,561	-1	TTGACTCAGCT	3.6Kb 5'of Gγ-globin start codon
5,271,193	5,271,183	-1	TTGACAAGGCA	Gγ-globin promoter**
5,276,118	5,276,108	-1	TTGACAAGGCA	Aγ-globin promoter**
5,265,437	5,265,487	-1	GTGACAAAGCG	360bp 3' of BGLT3
5,264,597	5,264,587	-1	CTGACTTTGCA	Ψβ-globin
5,264,499	5,264,489	-1	CTGACTGTGCA	Ψβ/δ intergenic region
5,258,538	5,258,548	+1	CTGACTGGGCA	Ψβ/δ intergenic region

*Chromosome 11 nucleotide sequence referenced from human reference sequence (GRCh37); +1, plus strand; -1, minus strand; BGLT3, β-globin locus transcript 3; **γ-globin ARE investigated in this study.

Supplementary Figures

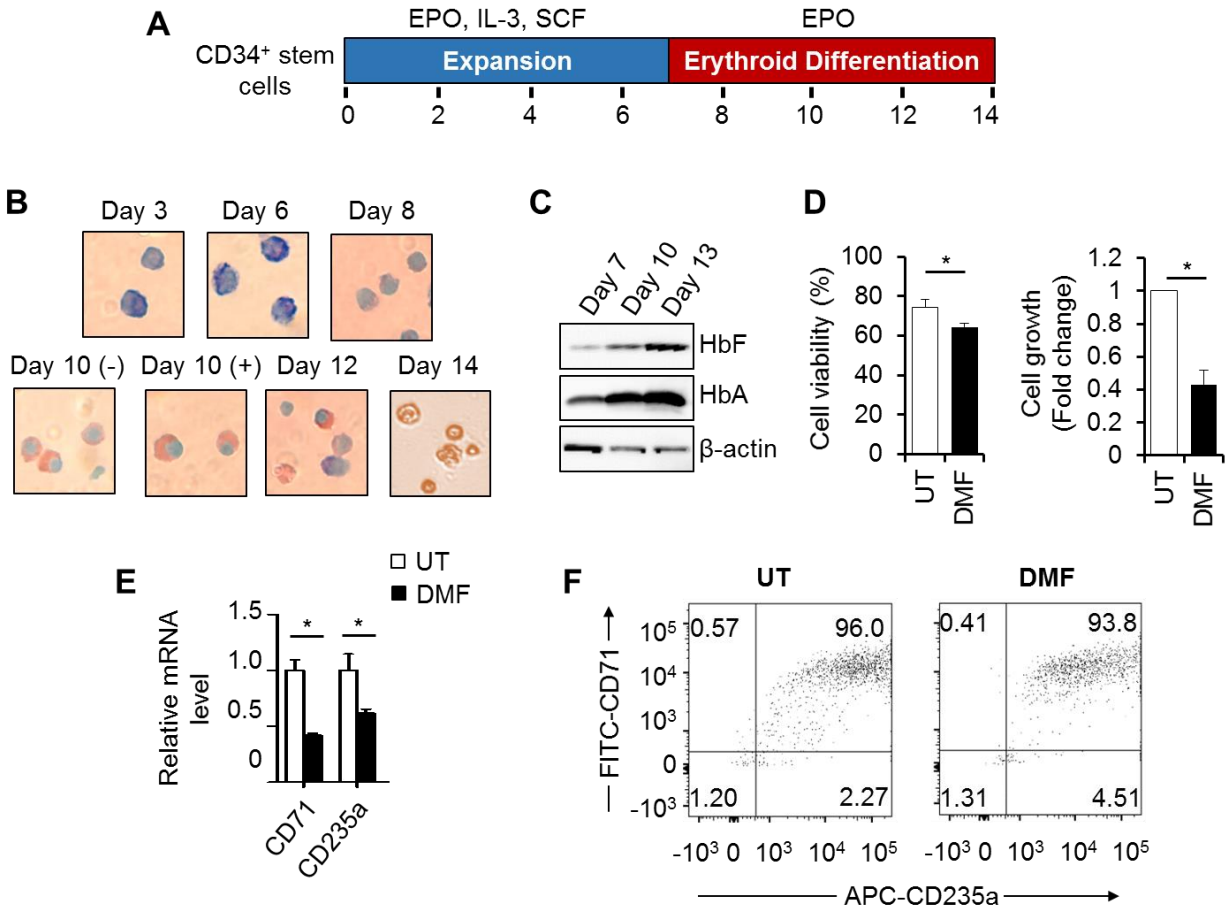

Figure S1. Generation of primary erythroid progenitors *in vitro* and DMF treatment. (A) Human bone marrow CD34$^+$ stem cells were cultured in a two-phase culture system. Briefly, cells were expanded during the first 7 days and differentiated from day 8 using the combination of growth factors indicated; erythropoietin (EPO), interleukin-3 (IL-3), and stem cell factor (SCF). **(B)** The morphology of erythroid progenitors were assessed by Giemsa staining on the days shown. Day 8 progenitors were treated with DMF for 48 hours and harvested at Day 10 for data analysis. Symbols: (-), no DMF treatment; (+), DMF treated. **(C)** Shown is a representative western blot gel of HbF, HbA and β-actin protein levels during erythroid differentiation. **(D)** The effects of DMF on cell viability and growth of erythroid progenitors. Day 8 CD34 erythroid progenitors (0.5x10^6/ml) were grown untreated (white bar) or treated with DMF (black bars) for

48 hours and cell viability (left graph) was determined by 0.4% Trypan blue exclusion and cell counts were conducted (right graph) at Day 10. The results are shown as the average ± standard error of the mean (SEM) from three independent experiments; *$p<0.05$. Values for untreated CD34 erythroid progenitors were normalized to one. **(E)** Expression of erythroid cell surface markers CD71 and CD235a in erythroid progenitors generated in liquid culture system were treated at day 8 with DMF (200 μM) for 48 hours. Reverse transcription-quantitative PCR (RT-qPCR) was conducted for untreated erythroid progenitors (white bars) or cells treated with DMF (black bars) to determine the mRNA levels. The expression of CD71 and CD235a mRNA in untreated erythroid progenitors was set to one after normalized to the internal control glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels. **(F)** Representative flow cytometry analyses on expression of cell surface marker CD71 and CD235a for erythroid progenitors treated in (E).

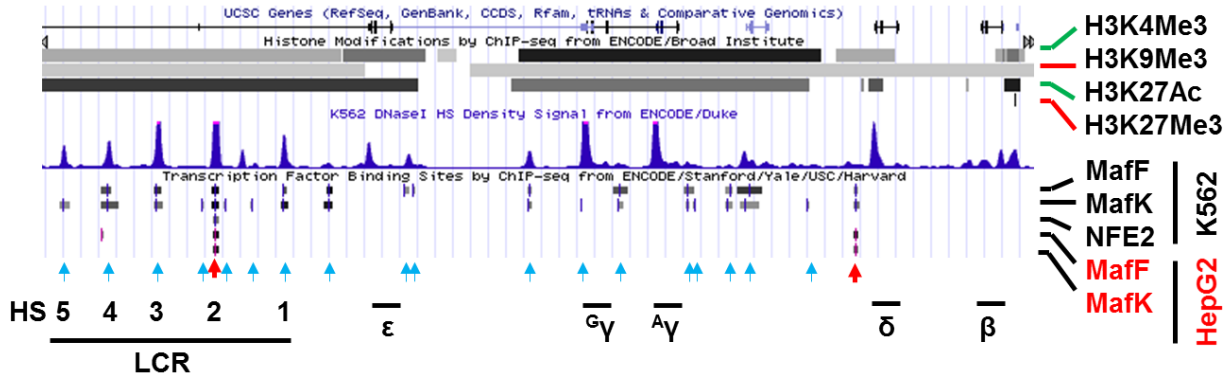

Figure S2. Binding of MAF across the *HBB* locus. ENCODE ChIP-seq data were downloaded from the UCSC server to determine MAF binding patterns across the *HBB* locus (https://genome.ucsc.edu/cgi-bin/hg). Data generated using K562 cells and HepG2 liver cancer cells are shown. The histone active chromatin marks H3K4Me3 (histone 3 lysine 4 trimethylation) and H3K27Ac (lysine 27 acetylation) and the repressive marks H3K9Me3 (histone 3 lysine 9 trimethylation) and H3K27Me3 (lysine 27 trimethylation) are shown by the black and gray horizontal lines. DNase I hypersensitivity (DNaseI HS) is represented by the blue peaks. The ENCODE data revealed significant small MAF protein MafF and MafK binding across the *HBB* locus in K562 cells. The ENCODE data was modified with predicted antioxidant response element (ARE) motifs (blue arrow) in *HBB* locus identified using JASPAR software and the general consensus sequence 5'-TGACnnnGC-3' (supplemental Table 2). Red arrows indicated the binding sites of MafF/K in both K562 and HepG2 cells. Note the overlap of predicted ARE motifs with MAF binding site.