Supplemental Appendix

Loss of NRF2 function exacerbates the pathophysiology of sickle cell disease in a transgenic mouse model

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Supplemental Material and Methods

Mice

The humanized Townes SCD transgenic (B6;129-*Hba*$^{tm1(HBA)Tow}$ *Hbb*$^{tm2(HBG1,HBB*)Tow}$/*Hbb*$^{tm3(HBG1,HBB)Tow}$/J)[1] and C57BL/6J NRF2 knockout (NRF2$^{-/-}$)[2] mouse were crossbred to generate the NRF2 knockout SCD mouse (SCD/NRF2$^{-/-}$) shown in Figure S1A. Mouse genotyping was performed with gene specific primers summarized in Supplementary Table S1. All animal studies were approved by the Augusta University Institutional Animal Care and Use Committee.

RNA analysis

Total RNA was isolated and analyzed as reported previously[3] for the human γ- and β^S-globin genes and mouse IL-1β, IL-6, IL-10, and TNFα genes; mouse 18s rRNA levels were used as an internal control (Supplemental Table S1). All mRNA levels were normalized to 18s rRNA levels before analysis.

Western Blot

Western blot analysis was performed with antibodies against HbF (51-7), HbA (37-8), NRF2 (H-300), catalase (H-300), ICAM-1 (G-5), VCAM-1 (E-10), and VEGF (C-1) from Santa Cruz Biotechnology as previously published.[3] Antibodies against NQO1 (ab80588, Abcam), HMOX1 (ab13248, Abcam), GCLC (ab41463, Abcam) and β-actin (A5316, Sigma) were purchased from the companies indicated.

Complete blood count and differential

Peripheral blood was collected in BD Vacutainer EDTA tubes by tail bleeding using protocols approved by the Augusta University Institutional Animal Care and Use Committee. Complete blood

count and differentials were completed on the Micros 60 CS/CT machine (HORIBA Medical/ABX Diagnostics, Irvine, CA) according to the manufacturer's protocol.

Flow cytometry

Flow cytometry was used to measure ROS using 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen) staining as reported previously.[4] Mouse peripheral blood samples were washed twice with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} before incubated with 5μM H_2DCFDA for 30 minutes at 37°C. Samples were washed with PBS and analyzed on a BD LSR-II flow cytometer for ROS level. The gating of RBCs was performed by forward scatter area lineage (FSC-A) *versus* side scatter area lineage (SSC-A) dot plot.[5] Subsequently, single cells were gated by forward scatter wide (FSC-W) *versus* FSC-A and the expression of the erythroid-specific marker Ter119 measured by staining with phycoerythrin conjugated rat anti-mouse Ter119 antibody (BD biosciences) (Figure S2). To measure the percentage of HbF positive cell (F-cells), erythroid progenitors were fixed with 1% formaldehyde and stained with FITC conjugated sheep anti-human HbF antibody (Abcam); isotype control IgG was used to detect non-specific staining for all flow cytometry studies. ROS and F-cell levels were quantified on a LSR-II flow cytometer (BD Biosciences).

Isolation of mouse hematopoietic progenitor cells from bone marrow

Single cell suspensions of bone marrow were prepared from 2-3 month old mice femur and processed with Ammonium-Chloride-Potassium red cell lysis buffer (Lonza). Subsequently bone marrow was processed with EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit (Stemcell Technologies Inc.) and the expression of HbF and cell surface markers including c-Kit, Sca1 and transferrin receptor (CD71) were determined by flow cytometry using FITC rat anti-mouse CD117 (c-kit) (BD biosciences), FITC rat anti-mouse CD71 (BD biosciences), FITC sheep anti-human fetal hemoglobin antibody

(Abcam), PE rat anti-mouse Ly-6A/E (Sca-1) (BD biosciences) and PE rat anti-mouse CD34 (BD biosciences) on a LSR-II flow cytometer (BD Biosciences).

In vitro RBC sickling analysis

Peripheral blood was collected by tail bleeding in BD Vacutainer EDTA tube and then washed with PBS. The cells were suspended in a 1:300 dilution in Iscove's Modified Dulbecco's Medium containing 10% fetal bovine serum, and incubated at 37^{o}C for 6 hours in normoxic (21% O_2) and hypoxic (1% O_2) conditions in a hypoxic chamber O_2 Tissue Culture Glove Box (Coy Laboratory Products, Grass Lake, MI). The blood samples were crosslinked with 4% formaldehyde for 10 minutes before transfer to room air. *In vitro* sickling of RBCs was quantified by changes in cell morphology by light microscopy. Bright field images (20× magnification) of RBC in PBS were acquired on an EVOS Cell Imaging Systems (Thermo Fisher Scientific).

Histopathology

Spleens were harvested immediately after mouse sacrifice and fixed with 10% buffered formalin. Tissue sections were stained with Hematoxylin & Eosin stain and image acquisition performed on an AxioVision Imager fluorescent microscope (ZEISS) supported with an AxioCam HRC camera (ZEISS). Images were captured at 5x or 20x magnifications and AxioVision REL 4.8 software was used for data analysis.

Statistical analysis

Data are presented as mean ± SD determined by Student's t-test; a p value <0.05 was considered significant.

References

1. Ryan TM, Ciavatta DJ, Townes TM. Knockout-transgenic mouse model of sickle cell disease. *Science*. 1997;278(5339):873-6.
2. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun*. 1997;236(2):313-22.
3. Zhu X, Li B, Pace BS. NRF2 Mediates γ-Globin Gene Regulation and Fetal Hemoglobin Induction in Human Erythroid Progenitors. *Haematologica*. 2017;102(8):e285-e288.
4. Hsiao CH, Li W, Lou TF, Baliga BS, Pace BS. Fetal hemoglobin induction by histone deacetylase inhibitors involves generation of reactive oxygen species. *Exp Hematol*. 2006;34(3):264-73.
5. Balmus G, Karp NA, Ng BL, Jackson SP, Adams DJ, McIntyre RE. A high-throughput in vivo micronucleus assay for genome instability screening in mice. *Nat Protoc*. 2015;10(1):205-15.

Supplementary Figures

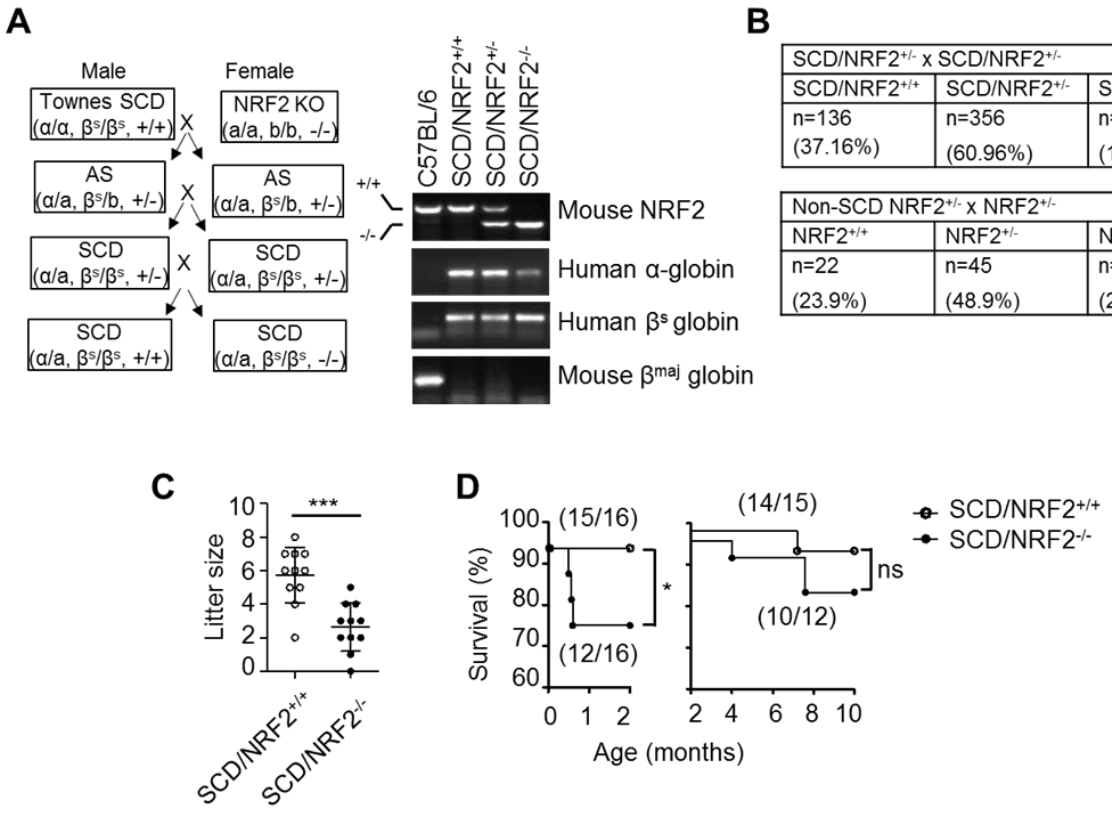

Figure S1. Cross-breeding approach to establish SCD/NRF2$^{-/-}$ mice. (A) Shown is the schematic of the breeding strategy used to cross the Townes SCD mouse with NRF2$^{-/-}$ mice (left) and PCR genotyping results to confirm SCD/NRF2$^{+/+}$ and SCD/NRF2$^{-/-}$ mice (right). Abbreviations: α, human α globin; a, mouse α globin; β^S, human β^S gene; b, mouse β-major globin gene. PCR genotyping was conducted for 3 loci including α-globin, β-globin, and NRF2 for each mouse. (B) The frequency of pups with the different genotypes after inbreeding SCD/NRF2$^{+/-}$ mice (top) and the frequency of pups for NRF2$^{+/+}$, NRF2$^{+/-}$ and NRF2$^{-/-}$ after inbreeding C57BL/6 NRF2$^{+/-}$ mice (bottom) are shown. (C) The average litter size was determined for both SCD/NRF2$^{+/+}$ (n=20) and SCD/NRF2$^{-/-}$ mice (n=18). (D) The Kaplan-Meier analysis was completed to determine postnatal survival probability of <1 month and 2-10 months old SCD/NRF2$^{-/-}$ and SCD/NRF2$^{+/+}$ mice (n=16 for each); *, $p<0.05$; ns, not significant.

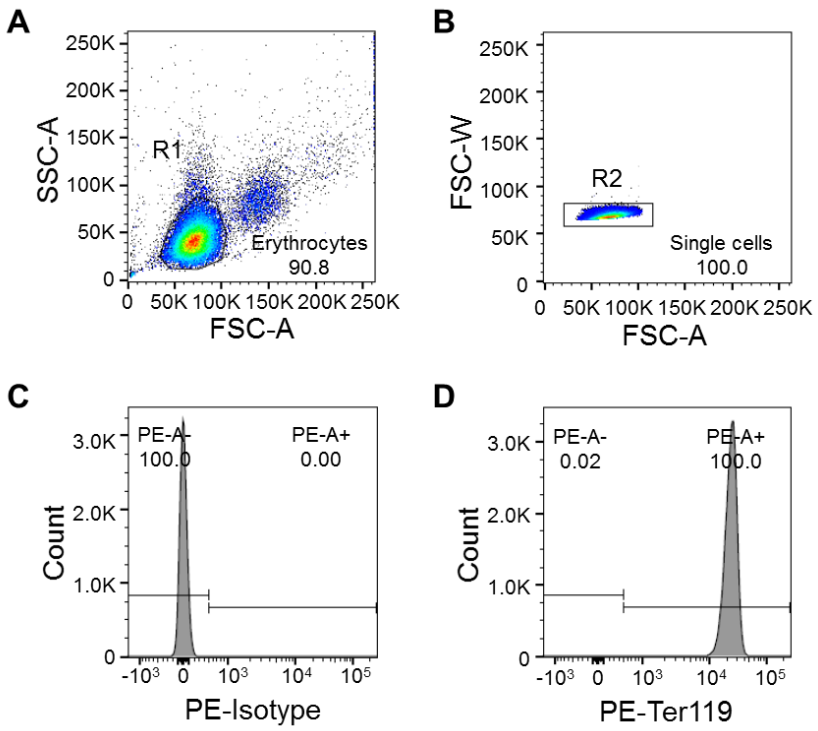

Figure S2. Flow cytometry analysis of RBC Ter119 expression. Mouse peripheral blood samples were washed with PBS and stained with PE-Ter119 antibody or isotype control antibody before analyzed by flow cytometry. (A) Erythrocyte cells were resolved by gating the R1 region on the side scatter area linear scale (SSC-A) *versus* forward scatter area linear scale (FSC-A). (B) Single cells were isolated by gating the R2 region on forward scatter width (FSC-W) *versus* FSC-A. (C-D) The gated cells (R1 and R2) were analyzed for Ter119 expression using PE-Isotype (C) or PE-Ter119 (D) antibody based on the intensity of fluorescence in the PE channel. The R1 and R2 regions gated erythrocyte cells were positive for Ter119 expression (PE-A+) (D).

Supplementary Table S1. Summary of primer sequences	
Genotyping primers	**5'→3'**
human α-globin gene forward primer	AGAGAAGTCCCACTCCAGCA
human α-globin gene reverse primer	AACACACACCAGGGAGAAGG
human β^S globin gene forward primer	TTGAGCAATGTGGACAGAGAAGG
human β^S globin gene reverse primer	AATTCTGGCTTATCGGAGGCAAG
mouse α1/ α2 globin gene forward primer	AGTGGGCAGCTTCTAACTATGC
mouse α1/ α2 globin gene reverse primer	GTCCCAGCGCATACCTTG
mouse β-major globin gene forward primer	TTGAGCAATGTGGACAGAGAAGG
mouse β-major globin gene reverse primer	ATGTCAGAAGCAAATGTGAGGAGCA
mouse NRF2 gene wild-type locus forward primer	GCCGCCTTTTCAGTAGATGGAGG
mouse NRF2 gene wild-type locus reverse primer	TGGACGGGACTATTGAAGGCTG
mouse NRF2 gene knockout locus forward primer	GCGGATTGACCGTAATGGGATAGG
mouse NRF2 gene knockout locus reverse primer	TGGACGGGACTATTGAAGGCTG
RT-PCR primers	**5'→3'**
human γ-globin forward primer	GGCAACCTGTCCTCTGCCTC
human γ-globin reverse primer	GAAATGGATTGCCAAAACGG
human β^s-globin forward primer	CTCATGGCAAGAAAGTGCTCG
human β^s-globin reverse primer	AATTCTTTGCCAAAGTGATGGG
mouse IL-1β (NM_008361) forward primer	CCCAAGCAATACCCAAAGAA
mouse IL-1β (NM_008361) reverse primer	CATCAGAGGCAAGGAGGAAA
mouse IL-6 (NM_031168) forward primer	TTCCATCCAGTTGCCTTCTT
mouse IL-6 (NM_031168) reverse primer	ATTTCCACGATTTCCCAGAG
mouse IL-10 (NM_010548) forward primer	GGACAACATACTGCTAACCGACTC
mouse IL-10 (NM_010548) reverse primer	AAAATCACTCTTCACCTGCTCCAC
mouse TNFα (NM_013693) forward primer	GCCTCTTCTCATTCCTGCTT
mouse TNFα (NM_013693) reverse primer	CACTTGGTGGTTTGCTACGA
mouse 18s rRNA (NR_003278) forward primer	AGTCCCTGCCCTTTGTACACA
mouse 18s rRNA (NR_003278) reverse primer	CGATCCGAGGGCCTCACTA