SUPPLEMENTARY APPENDIX

Circulating iron levels influence the regulation of hepcidin following stimulated erythropoiesis

Cornel S.G. Mirciov,[1,2] Sarah J. Wilkins,[1] Grace C. C. Hung,[1] Sheridan L. Helman,[1,3] Gregory J. Anderson[1,2,4] and David M. Frazer[1,2]

[1]Iron Metabolism Laboratory, QIMR Berghofer Medical Research Institute, Herston; [2]School of Medicine, The University of Queensland, St Lucia; [3]School of Biomedical Sciences, Queensland University of Technology, Gardens Point and [4]School of Chemistry and Molecular Bioscience, The University of Queensland, St Lucia, Australia.

 doi:10.3324/haematol.2017.187245

Received: November 20, 2017.

Accepted: May 16, 2018.

Pre-published: June 4, 2018.

Correspondence: david.frazer@qimrberghofer.edu.au

Supplementary Methods

Liver and serum iron levels were determined as previously described[1, 2]. Transferrin saturation was determined by first measuring the total iron level and the unbound iron binding capacity (UIBC) of the serum. The total iron binding capacity (TIBC) of the serum was then calculated by adding the total iron and the UIBC. Finally, the total iron was divided by the TIBC x 100 to give the percentage transferrin saturation. Transferrin species were quantified from plasma samples by urea PAGE analysis as previously described[3, 4]. The primary antibody used was a goat anti-mouse transferrin IgG (1:10000, Alpha Diagnostic International, TX) and the secondary was a donkey anti-sheep/goat IgG antibody, HRP conjugate (1:10000, Chemicon, Boronia, Australia).

Analysis of gene and protein expression

Gene expression analysis was carried out on bone marrow, spleen and liver tissue as previously described[2]. Primer sequences are listed in Table S1. The level of phosphorylated SMADs 1, 5 and 8 in liver tissue was determined by Western blotting as previously described[5]. The primary antibodies used were rabbit polyclonal antibodies to either phosphorylated SMAD 1/5/8 (1:1000; Cell Signaling Technology, Danvers, MA, USA), SMAD1 (1:250; Invitrogen) or ACTB (β-actin; 1:1500; Sigma-Aldrich, Castle Hill, NSW, Australia) and the secondary antibody was a goat anti-rabbit horseradish peroxidase secondary antibody (1:1000; Merck, Kilsyth, Australia).

References

1. Frazer DM, Wilkins SJ, Darshan D, Mirciov CSG, Dunn LA, Anderson GJ. Ferroportin Is Essential for Iron Absorption During Suckling, But Is Hyporesponsive to the Regulatory Hormone Hepcidin. Cell Mol Gastroenterol Hepatol. 2017;3(3):410-421.

2. Mirciov CS, Wilkins SJ, Dunn LA, Anderson GJ, Frazer DM. Characterization of Putative Erythroid Regulators of Hepcidin in Mouse Models of Anemia. PLoS One. 2017;12(1):e0171054.

3. Frazer DM, Inglis HR, Wilkins SJ, et al. Delayed hepcidin response explains the lag period in iron absorption following a stimulus to increase erythropoiesis. Gut. 2004;53(10):1509-1515.

4. Wilkins SJ, Frazer DM, Millard KN, McLaren GD, Anderson GJ. Iron metabolism in the hemoglobin-deficit mouse: correlation of diferric transferrin with hepcidin expression. Blood. 2006;107(4):1659-1664.

5. Wallace DF, Summerville L, Crampton EM, Frazer DM, Anderson GJ, Subramaniam VN. Combined deletion of Hfe and transferrin receptor 2 in mice leads to marked dysregulation of hepcidin and iron overload. Hepatology. 2009;50(6):1992-2000.

Table S1. Primer sequences used for quantitative polymerase chain reaction.

Gene abbreviation	Full gene name	Primer sequence
Erfe	*Erythroferrone*	Forward - CCAGGCCCCTTTATCCCATC
		Reverse - GTGCTCCAGATGGCTCTCTC
Gypa	*Glycophorin A*	Forward - GTGATGGCAGGGATTATCGGA
		Reverse - CACTGTTGTCACCACCCTCA
Hamp1	*Hepcidin antimicrobial peptide 1*	Forward - CCTGAGCAGCACCACCTATC
		Reverse - TGCAACAGATACCACACTGGG
Hprt	*Hypoxanthine guanine phosphoribosyl transferase*	Forward - GGACTGATTATGGACAGGA
		Reverse - GAGGGCCACAATGTGATG
Tfr1	*Transferrin receptor 1*	Forward - TCATGAGGGAAATCAATGATCG
		Reverse - CCCCAGAAGATATGTCGGAAAG

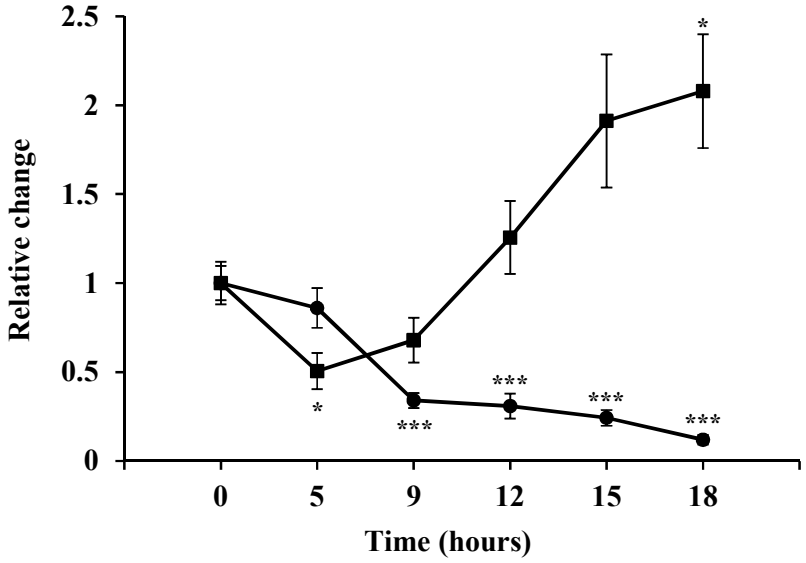

Figure S1. Relative changes to *Hamp1* expression and diferric transferrin levels following erythropoietin injection in mice.

Six week old male C57BL/6 mice were euthanized 0, 5, 9, 12, 15 or 18 hours following the intravenous injection of 10U/g body weight human erythropoietin and tissues were taken for analysis. Hepatic *Hamp1* expression (●) and relative diferric transferrin levels (■) were determined for each time point (as represented in Figures 1A and 3D). Gene expression levels were calculated relative to the general housekeeping gene *Hprt* and are expressed as a proportion of the values at 0 hours. The relative diferric transferrin levels represent the percentage of transferrin in the diferric form expressed as a proportion of the values at 0 hours. The data represent mean ± SEM. Statistical significance is shown relative to the 0 hours group. *$P < 0.05$; ** $P < 0.01$; ***$P < 0.005$.