Iron deficiency anemia in cyclic GMP kinase knockout mice

Mice with a global inactivation of the cyclic GMP kinase I (cGKI) gene (cGKI$^{-/-}$) and mice that express cGKIα or cGKIβ in all smooth muscles on a cGKI$^{-/-}$ background[1] (cGKI RM mice) have a bleeding duodenal ulcer[2] and anemia of unknown cause.[3,4] Immunocytochemistry of bone marrow indicated that macrophages but not erythroblasts express cGKI.[1] In contrast, it was found that erythroblasts and erythrocytes purified by immunomagnetic selection using labeled anti-Ter119 MicroBeads followed by magnetic cell sorting are cGKI positive.[3] cGKI deleted erythrocytes showed a marked increase in annexin V binding, indicating phosphatidylserine (PS) exposure at the outer membrane leaflet, a hallmark of suicidal erythrocyte death or eryptosis.[5] Eryptosis caused faster erythrocyte clearance *in vivo*. Therefore, it was suggested that the observed anemia was induced by the rapid erythrocyte clearance in the spleen.[3]

We investigated the discrepancy concerning cGKI expression in erythrocytes/erythroblasts[1,3] using Western blots of purified erythrocytes/erythroblasts (Figure 1). These experiments were carried out in three independent laboratories using different methods for purification of the erythrocytes. None of the groups were able to detect significant amounts of cGKI protein in wild-type (WT) erythrocytes or in TER119 purified erythrocytes/erythroblast. Although the negative Western blots did not rule out that the stability of the erythrocytes from cGKI$^{-/-}$ mice might be reduced, these results suggested that the anemia of cGKI$^{-/-}$ mice may be due to a different cause, e.g. bleeding of duodenal ulcers.[2]

Reinvestigation of the occurrence of blood loss in the feces of cGKI$^{-/-}$ mice showed that 9 out of 10 mice had blood present in their feces (*Online Supplementary Figure S1*). Blood loss was stopped by feeding the proton pump inhibitor (PPI) esomeprazole to cGKI$^{-/-}$ and cGKI RM mice (*Online Supplementary Figure S1*). The blood loss was severe, because cGKI$^{-/-}$ and cGKI RM mice had all the signs of hyper-regenerative anemia, namely, decreased erythrocyte numbers and hemoglobin levels and a decreased hematocrit and markedly increased reticulocyte count (Figure 2). These values were normalized by PPI treatment. The mean corpuscular volume (MCV) and red blood cell distribution width (RDW-CV) were elevated in the untreated mice, but returned to normal following PPI treatment (Figure 2). The MCV was higher than expected, because this value is calculated by the size of the red blood cells that include immature erythrocytes. The increased reticulocyte counts could also be indicative of hemolytic anemia in cGKI$^{-/-}$ mice, however, this possibility has been ruled out by Föller and colleagues.[3]

No iron could be detected in the spleen of untreated cGKI$^{-/-}$ and cGKI RM mice (*Online Supplementary Figure S2*). As expected with chronic regenerative anemia, spleen size and spleen weight (Figure 3A) were considerably increased. Histological inspection indicated an increased erythrocyte content of the spleen, as reported by Föller and colleagues.[3] The spleen size and spleen weight were normalized by treatment with the PPI (Figure 3A) in the cGKI$^{-/-}$ and cGKI RM mice. The iron content of the spleen was increased by either PPI treatment or iron injection, and was normalized by the com-

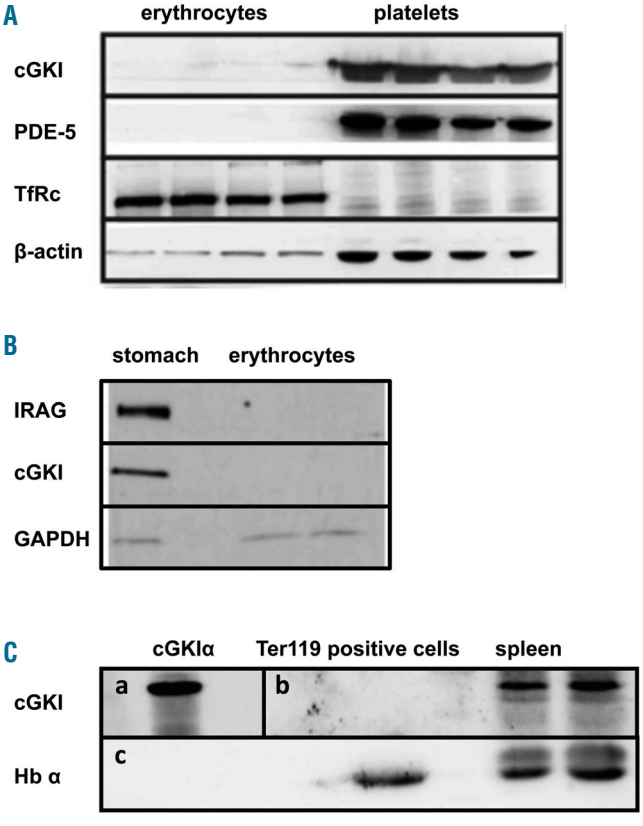

Figure 1. Western blot of cGKI in WT Erythrocytes. (A,B,C) cGKI Western blots were performed in three different laboratories using different methods. Details of preparation of cells are given in *Online Supplementary Methods*. (A) 30 (platelets) and 40 (erythrocytes) μg protein lysates were loaded per slot. Proteins of slots 1 and 5, 2 and 6, 3 and 7, and 4 and 8 were from the same mouse preparation. A transferrin receptor 1 (TfRc) specific antibody was used to identify the erythrocytes. Equal loading of the gel was demonstrated by detecting β-actin. The cGMP-degrading phosphodiesterase 5 (PDE-5) was only present in platelets allowing for further differentiation between erythrocyte and platelet lysates. (B) Erythrocytes were purified by differential centrifugation from wild-type and cGKIα RM mice. 70 μg protein was loaded per slot. As control, 70 μg protein from WT stomach were analyzed with antibodies directed against cGKI, inositol 1,4,5-trisphosphate receptor associated cGMP kinase substrate (IRAG) or GAPDH. (C) 45 μg protein from erythrocytes purified by immunomagnetic selection using magnetically labeled Anti-Ter119 MicroBeads were loaded per slot. 100 μg WT spleen proteins were loaded per slot. In Figure 1Ca 5 ng purified cGKIα was loaded on the slot. For better comparison, Western blots were developed for 4 sec (Figure 1Ca), 30 sec (Figure 1Cb), and 1 sec (Figure 1Cc). Hbα: hemoglobin α-chain.

bination of PPI and iron injection (*data not shown*).

The severe iron store deficiency should also be reflected by a decreased plasma iron concentration. Measurement of plasma iron showed that cGKI$^{-/-}$ and cGKI RM mice have a severe decrease in plasma iron concentration that is reversed by treatment with PPI (Figure 3B). Iron resorption depends on two proteins present in the duodenum, the divalent metal ion transporter 1 and ferroportin.[6] Ferroportin releases iron on the basolateral side of the enterocyte to ceruloplasmin and transferrin. The ferroportin concentration is regulated by the liver protein hepcidin. Hepcidin transcription is subsequently regulated by the BMP receptor and SMAD proteins.[6,7] High hepcidin concentrations decrease the concentration of ferroportin. Therefore, hepcidin concentration should be low in iron deficiency. In line with these established feedback mechanisms, the liver hepcidin (*HAMP*) mRNA concentration was low in cGKI$^{-/-}$ mice (Figure 3C), but increased with PPI treatment and iron injection. The combination of iron injection and PPI normalized the hepcidin concentration (Figure 3C).

In agreement with the known regulation in anemia, the transcription of the transferrin receptor (Figure 1A and 3D) was upregulated in cGKI$^{-/-}$ and cGKI RM mice. From these results, and the negative staining of the iron stores in spleen (*Online Supplementary Figure S2*), we expected that the cGKI$^{-/-}$ mice would have a very low concentration of the iron storage protein ferritin in spleen and liver. Accordingly, the ferritin light chain (FLC) was not detected in spleen extracts from cGKI$^{-/-}$ mice at baseline (*Online Supplementary Figure S3A and S3B*). The ferritin light chain was easily detectable after PPI treatment or after injection of iron (*Online Supplementary Figure S3A and S3B*). The decrease of the ferritin light chain was also detected in liver extracts (*Online Supplementary Figure S3C and S3D*), although the effect of cGKI deletion was less dramatic in liver than in spleen (*Online Supplementary Figure S3C and S3D*). PPI treatment normalized the liver ferritin light chain concentration (*Online Supplementary Figure S3C and S3D*).

The cGKI$^{-/-}$ and the cGKI RM mice have been reported to have an elevated IL-6 concentration.[4,8] IL-6 stimulates hepcidin transcription.[6] Elevated IL-6 concentrations are observed in inflammatory states, a known cause for anemia that responds poorly to iron or PPI treatment.[9] Because red blood cell counts, FLC levels, spleen and liver iron pools and many other parameters in the cGKI gene-targeted mouse models were sensitive to the PPI and iron treatment regimens, it seemed unlikely that the observed anemia was associated with chronic inflammation and/or

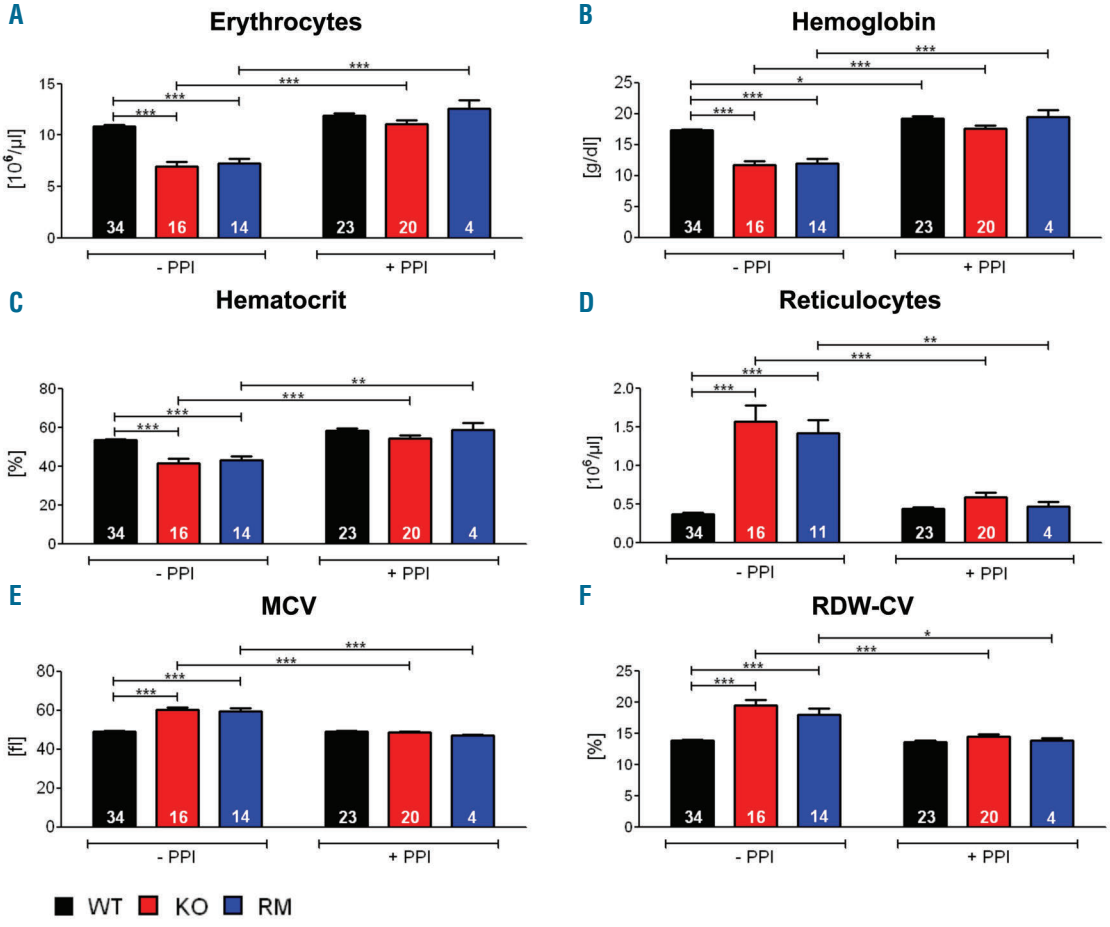

Figure 2. Effect of PPI on erythrocytes, hemoglobin, reticulocytes, mean corpuscular volume, and red blood cell distribution width (RDW-CV). Blood was taken from individual 6 to 12 week old mice. The number of mice is given within each column. Mice were not treated (-PPI) or treated with PPI (+PPI). A) erythrocytes; B) hemoglobin; C) hematocrit; D) reticulocytes; E) mean corpuscular volume (MCV); F) red blood cell distribution width (RDW-CV). ***P<0.001; **P<0.01; *P<0.05.

acute infection. We further investigated if an infection might cause the observed anemia phenotype by measuring IL-6 plasma levels (*Online Supplementary Figure S4*). As expected, IL-6 levels were elevated in untreated cGKI$^{-/-}$ mice, and its concentration was not affected by PPI treatment. The cGKI$^{-/-}$ and cGKI RM mice had anemia caused by bleeding intestinal ulcers. All anemia parameters were normalized by treatment of the animals with PPI, because PPI stopped the intestinal bleeding. Moreover, anemia indicators were ameliorated by iron injection, but the most efficient treatment was a combination of PPI plus iron injection. These results support the notion that the cGKI$^{-/-}$ mice have classical hyper-regenerative anemia due to chronic blood loss. A shortened lifespan of the cGKI$^{-/-}$ erythrocytes seems to be unlikely, since we were unable to identify the cGKI protein in erythrocytes. cGKI is highly expressed in platelets,[10] which may contaminate erythrocyte preparations.

Esomeprazole is a very specific inhibitor of the gastric H^+/K^+ ATPase.[11] H^+/K^+ ATPase is highly expressed in the gastric mucosa and has also been detected at very low concentrations in the human larynx, submandibular gland[12] and in pancreatic duct cells.[13] The gastric H^+/K^+ ATPase has not been found in leucocytes and bone marrow.[14] Importantly, none of the tissues expressing H^+/K^+ ATPase are known to be involved in erythrocyte maturation. Therefore, it is extremely unlikely that esomeprazole affected the anemia parameters by an effect outside of the stomach, i.e. by an unexpected effect on the red blood cell lineage.

The likely cause for the bleeding ulcer has been reported.[2] cGKI$^{-/-}$ mice are unable to neutralize the stomach acid in the duodenum.[2] The inhibition of gastric proton secretion by PPI results in a neutral gastric fluid, allowing for the healing of the ulcer and thereby stopping the blood loss. This was sufficient to reverse the severe anemia in cGKI$^{-/-}$ and cGKI RM mice. Therefore, we conclude that chronic intestinal bleeding, and not the decreased lifespan of erythrocytes due to eryptosis, is the major cause of anemia in these animals. Remarkably, the reversal of anemia prolongs the survival of the cGKI$^{-/-}$ mice,[2] suggesting that the premature death of cGKI$^{-/-}$ mice is at least partially caused by severe anemia.

These results support the notion that the cGMP/cGKI signaling system prevents the induction of duodenal ulcers and iron deficiency anemia. The inability to secrete bicarbonate in the duodenum was not caused by the loss of the cGKI protein in peripheral tissues, but by the loss of cGKI in the central nervous system (CNS), most likely in the nucleus tractus solitarius (NTS).[2] The NTS is present in the medulla oblongata and connects the afferent *N. vagus* with the efferent *N. vagus*. We suggest consider-

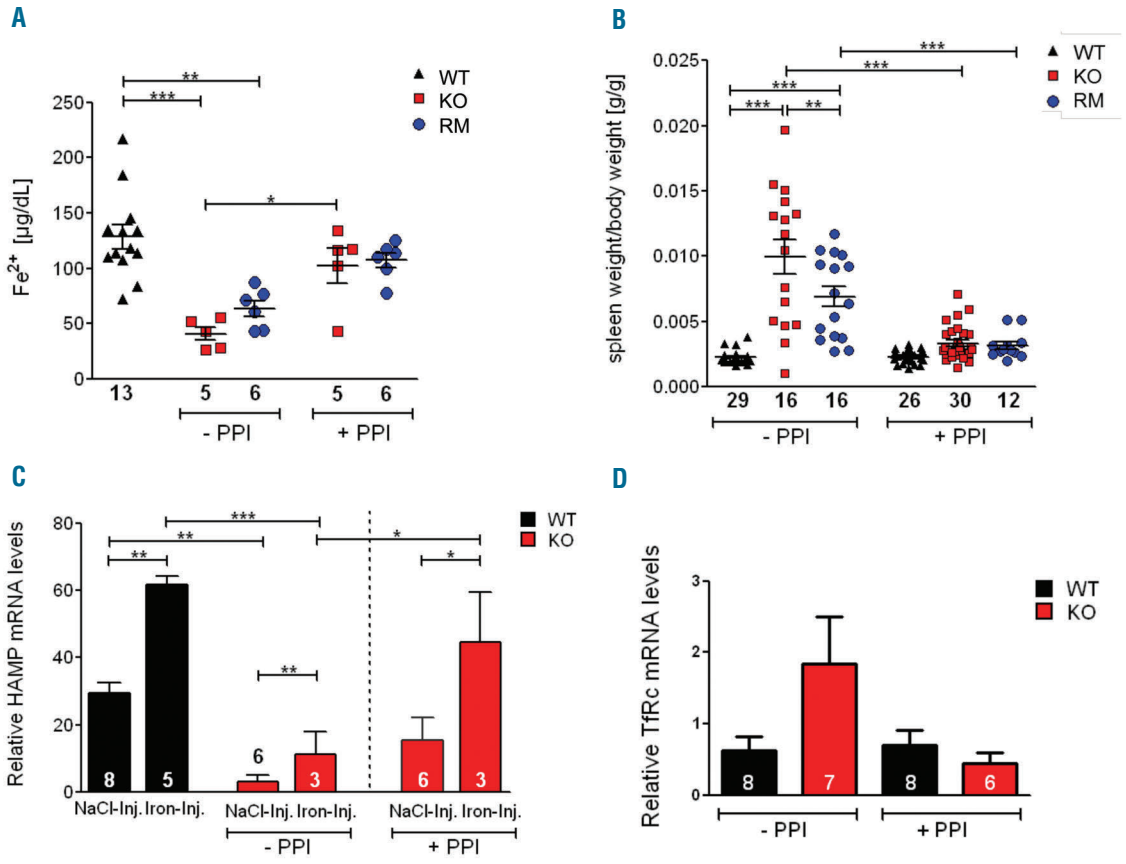

Figure 3. Plasma iron concentration (A), **spleen weight** (B), **and quantitative real-time PCR of liver *HAMP*** (C) **and transferrin receptor 1** (D) **mRNA.** A) Plasma iron and B) spleen weight were measured before (-PPI) and after (+PPI) treatment with PPI. The number of mice is given below each scatter plot. C) mRNA of liver hepcidin. cGKI$^{-/-}$ mice were injected i.p. with physiological sodium chloride (NaCl-Inj.) or with Fe^{3+} for 12 days (see *Online Supplementary Methods*). WT mice were not treated with PPI. D) mRNA of liver transferrin receptor 1 (TfRc). mRNA was extracted from the liver of WT and cGKI$^{-/-}$ (KO) mice. mRNA levels for hepcidin and transferrin receptor 1 were normalized to mRNA levels for GAPDH. Mice were not treated (-PPI) or treated (+PPI) with PPI. The number of mice is given within the columns. ***P<0.001; **P<0.01; *P<0.05.

ing an altered NO/sGC/cGMP/cGKI signaling complex regulation as a cause of duodenal ulcer development. This paper adds anemia, caused by a lack of cGKI, to the growing number of intestinal diseases caused by a dysfunction in the NO/sGC/cGMP/cGKI or cGKII signaling complex.

Elisabeth Angermeier,[1] Katrin Domes,[1] Robert Lukowski,[2] Jens Schlossmann,[3] Birgit Rathkolb,[4,5] Martin Hrabě de Angelis,[4,6,7] and Franz Hofmann[1]

[1]Institut für Pharmakologie und Toxikologie, Technische Universität München; [2]Pharmacology, Toxicology, and Clinical Pharmacy, Institute of Pharmacy, Universität Tübingen; [3]Pharmacology and Toxicology, Institute of Pharmacy, Universität Regensburg; [4]German Mouse Clinic, Institute of Experimental Genetics, Helmholtz Zentrum München, Neuherberg; [5]Institute of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians-Universität München; [6]Chair of Experimental Genetics, School of Life Science Weihenstephan, Technische Universität München, Freising, and [7]German Center for Diabetes Research (DZD), Neuherberg, Germany

Funding: The experimental work was supported by grants from Deutsche Forschungsgemeinschaft, Fond der Chemischen Industrie and the German Federal Ministry of Education and Research (Infrafrontier grant 01KX1012).

Acknowledgments: we want to thank S. Kaidel and S. Crowley for expert technical help.

Correspondence: Franz.Hofmann@mytum.de

doi:10.3324/haematol.2015.137026

Key words: cyclic GMP (cGMP), erythrocyte, gene knockout, H+/K+−ATPase, hemoglobin, protein kinase G (PKG).

The online version of this paper contains a Supplementary Appendix.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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