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Investigating the real role of HIF-1 and HIF-2 in iron recycling by macrophages

Most of the iron required for erythropoiesis is acquired by heme iron recycling of senescent erythrocytes by tissue macrophages (mainly spleen, bone marrow and liver macrophages). This process, called erythrophagocytosis (EP), is essential for mammalian iron homeostasis. Perturbations in EP occur in several diseases, including anemia of chronic disorders, hemochromatosis and thalassemia.[1]

The senescent red blood cell is internalized by phagocytosis and degraded within acidified early phagosomes. Heme is transported into the cytosol by the recently identified heme transporter HRG1 (Heme-responsive gene 1).[2] Heme is then catabolized by heme oxygenase 1 (HO-1) leading to the release of iron. DMT-1 (Divalent Metal Transporter-1) and natural resistance-associated macrophage protein 1 (Nramp1), play essential roles in heme iron recycling[3,4] but their functional roles remain to be determined. Iron is released from macrophages by the iron exporter ferroportin (FPN) or stored within ferritin. During EP, these different genes, such as HO-1 and FPN, are regulated at post-transcriptional level (through the

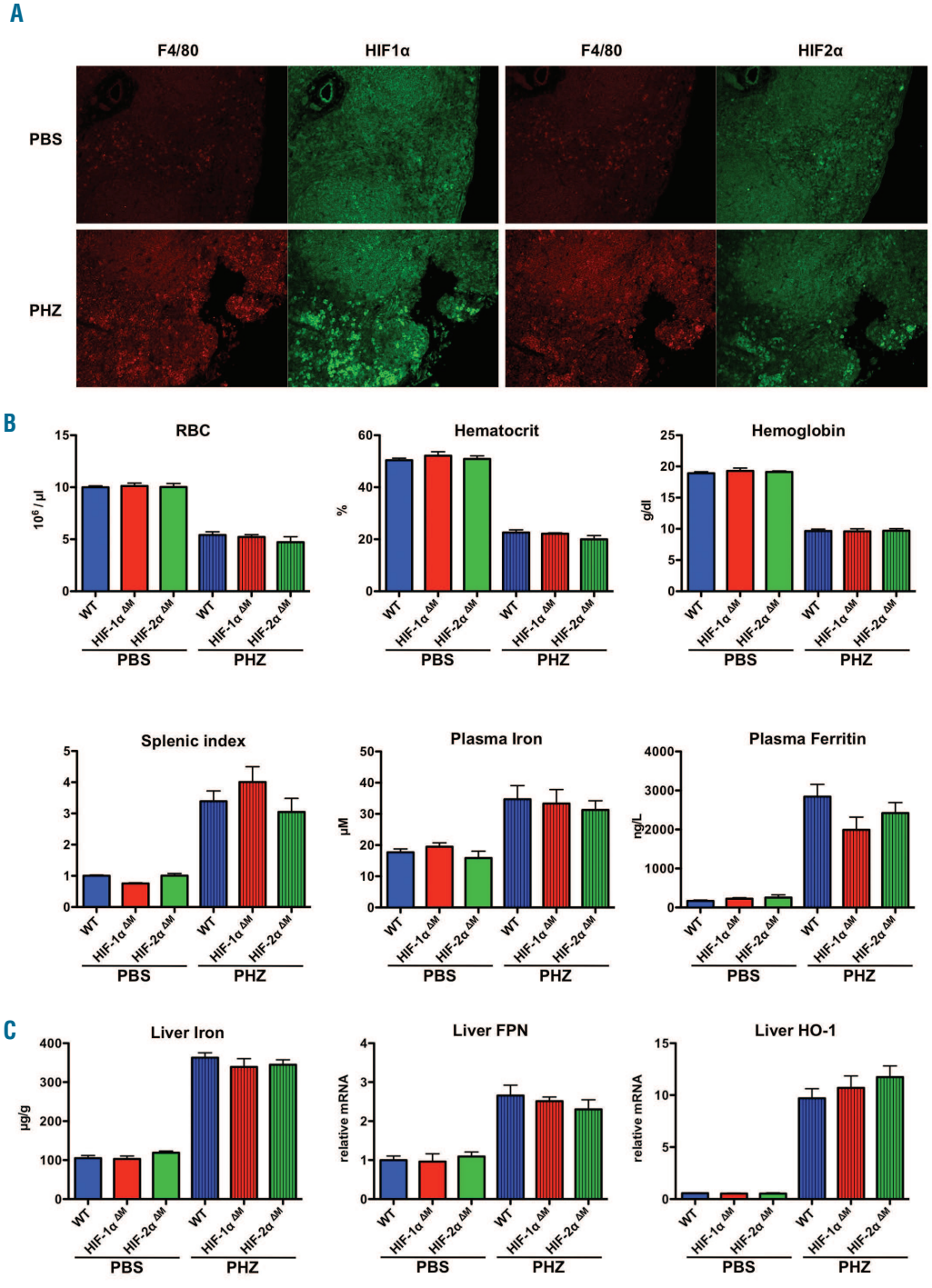


Figure 1. Iron homeostasis parameters in WT, HIF-1$\alpha^{\Delta M}$ and HIF-2$\alpha^{\Delta M}$ mice treated with PBS or PHZ. 12 week-old WT, HIF-1α^{Δ}M or HIF-2$^{\Delta}$M male mice (n ≥ 4) were injected for two consecutive days with 50 mg/kg of PHZ or PBS and were killed 48 h after the last injection. The mice were maintained on an iron deficient diet immediately after the first injection to avoid a compensatory increase in dietary iron absorption. (A) HIF-1, HIF-2 and F4/80 immunofluorescence on PBS or PHZ-treated WT mice. (B) Red blood cells (RBC), hemoglobin, splenic index (defined as the spleen/body weight ratio), plasma iron, ferritin levels and (C) liver Iron, liver FPN and HO-1 mRNA levels (by Q-PCR normalized to cyclophilin A) in WT and HIF-1α^{Δ}M or HIF-2$^{\Delta}$M mice treated with PBS or PHZ.

Iron Regulatory Proteins), and/or at a transcriptional level by heme through a pathway involving Bach1 (BTB and CNC homology 1)/Nrf2 (nuclear factor erythroid 2-related factor 2).[5]

Hypoxia and its cellular mediators (hypoxia inducible factors: HIF-1 and HIF-2) have been shown to transcriptionally regulate essential genes involved in EP (e.g. HO-1, DMT-1, FPN)[6-8] in a number of cell lines or tissues and have, therefore, been suggested to have a role in erythrophagocytosis,[9-11] a concept that can be read in a number of recent reviews, with no associated references. Nevertheless, the physiological role of HIF-1 and HIF-2 in iron recycling of erythrocytes has never been investigated. We, therefore, felt it was important to establish the 'real' role of HIF in iron recycling based on scientific data and not extrapolation from other reviews.

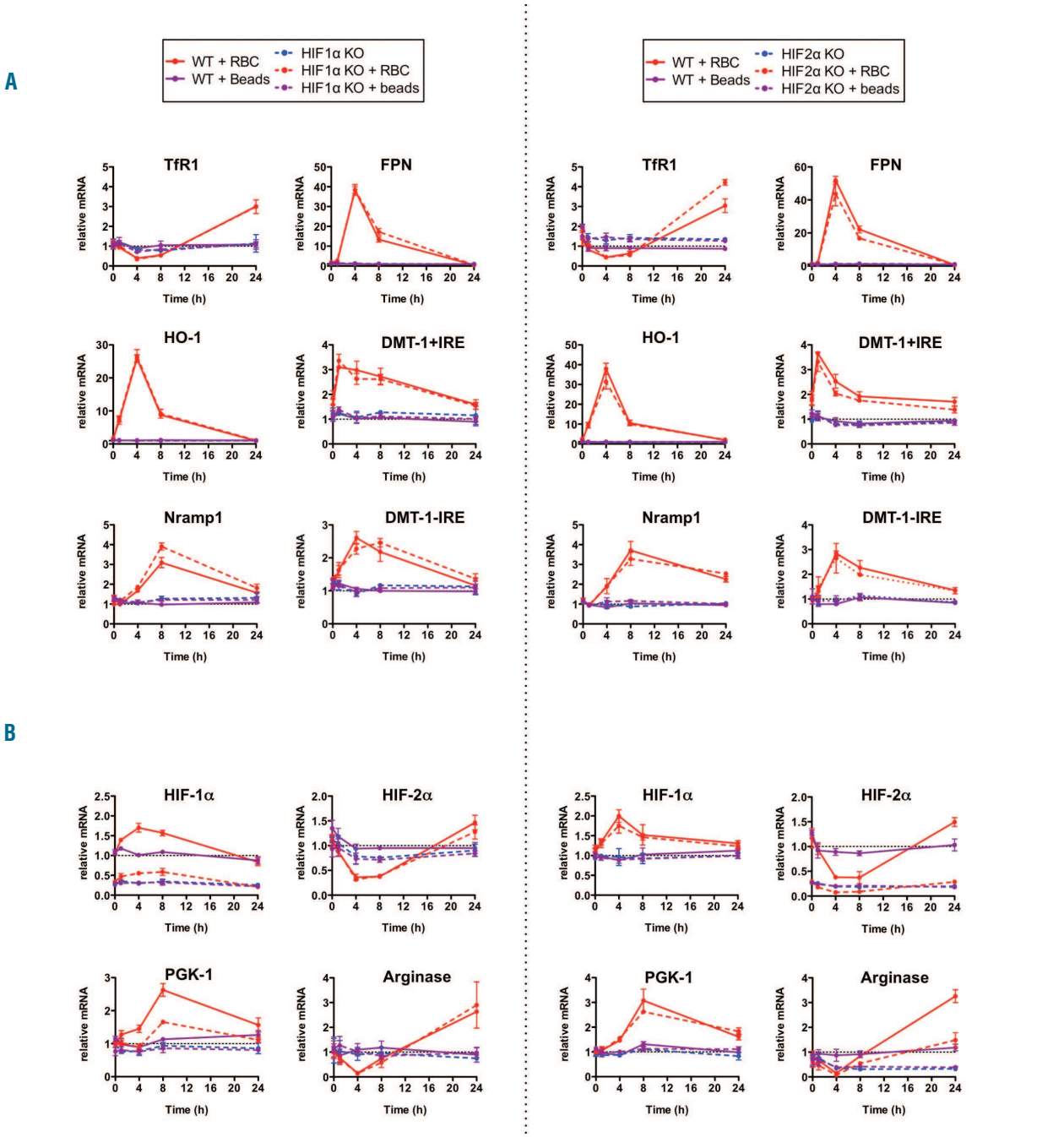


Figure 2. mRNA expression patterns of iron recycling genes after EP in HIF-1 or HIF-2 deficient BMDMs. Q-PCR time course analysis of (A) TfR1, FPN, HO-1, DMT-1+IRE, DMT-1-IRE, Nramp1, (B) HIF-1α, HIF-2 α, PGK-1, Arginase mRNA levels in WT, HIF-1 (HIF-1α KO) or HIF-2 deficient (HIF-2α KO) BMDMs incubated or not (-) with either latex beads (purple line) or RBCs (pink line). Red blood cell aging was achieved by incubating RBCs with a calcium ionophore solution. BMDMs were incubated with latex beads or RBCs (ratio 100 RBCs/BMDM) for 1 hour, then incubated 5 min in a hypotonic solution to lyse non-ingested RBCs and washed twice with PBS. Values were normalized to WT. RNA extraction, reverse transcription and quantitative PCR have been previously described.[8] All samples were normalized to the threshold cycle value for cyclophilin A. Sequences of the primers used are available upon request. n=3 per time point. Statistical values are represented in *Online Supplemental Figure S2*.

We found that HIF-1 and HIF-2 were stabilized in the macrophages of the spleen in conditions of increased erythropoiesis due to acute hemolytic anemia induced by phenylhydrazine (PHZ) (Figure 1A). To determine the impact of the deletion of macrophage HIF-1 and HIF-2 in iron recycling *in vivo*, we evaluated several parameters of iron homeostasis in HIF-1$^{lox/lox}$-LysM Cre (HIF-1$^{\Delta M}$),[12] HIF-2$^{lox/lox}$-LysM Cre (HIF-2$^{\Delta M}$)[13] mice and control littermates in basal or PHZ conditions. We first confirmed the decrease in HIF-1 and HIF-2 expression in the respective conditional knockout mice and validated the lack of compensatory mechanism (*Online Supplementary Figure S1A*). At steady state, the red blood cell, hematocrit, hemoglobin, plasma iron and ferritin levels, as well as the splenic indices were similar between WT and mutant mice (Figure 1B). Two days after PHZ treatment, the expected significant decrease of hematologic parameters, as well as the increased splenic index, ferritin (Figure 1B) and liver iron (Figure 1C) levels were observed similarly in WT and KO mice. The increased expression of liver HO-1 and FPN, two key genes involved in iron recycling, was identical between WT and mutant mice (Figure 1C). Importantly, the increased plasma iron, underlying increased iron recycling was intact in the KO mice (Figure 1B). The recovery from anemia was identical in WT and mutant mice (*Online Supplementary Figure S1B*).

We next used a characterized cellular model of iron recycling by macrophages,[14] which tends to mimic the natural clearance of senescent RBCs, to investigate more thoroughly whether this lack of effect was due to the inability of HIF-1 and HIF-2 in macrophages to regulate key genes involved in EP. Primary cultures of murine bone-marrow derived macrophages (BMDMs) were incubated with latex beads or artificially aged murine RBCs. Latex beads had no effects on any tested genes by quantitative PCR, showing that phagocytosis itself was not able to elicit gene expression responses (Figure 2). Four hours after the incubation of senescent RBCs, TfR1 mRNA levels were significantly decreased in BMDMs, concomitant to an increase in FPN and HO-1, indicating an increased iron content, as previously described[14,15] (Figure 2A). DMT-1+IRE mRNA levels were maximally induced as early as one hour after EP, while Nramp-1 accumulation was delayed, peaking at 8 h. Unexpectedly, after EP, we found an increase in HIF-1 expression, reaching maximal levels at 4 h and returning to basal levels at 24 h. Conversely, HIF-2 mRNA levels decreased maximally at 4 h (Figure 2B). To determine the role of HIFs in the transcriptional regulation of these iron recycling genes, we derived macrophages from HIF-1$^{\Delta M}$ and HIF-2$^{\Delta M}$ mice. The levels of HIF-1α and HIF-2 α mRNAs were markedly decreased in the macrophages deficient for HIF-1α and HIF-2α, respectively (Figure 2B). Neither the deletion of HIF-1 nor HIF-2 modified the expression of any tested genes involved in iron recycling (Figure 2A). Importantly, the response of prototypic HIF-1 and HIF-2 target genes, such as PGK-1 and Arginase, was significantly decreased in HIF-1 and HIF-2 deficient macrophages, respectively. Finally, deletion of one isoform did not affect the expression of the other, ruling out a putative compensatory mechanism.

In conclusion, in contrast to other cells or tissues, macrophage HIF-1 and HIF-2 are not required in the regulation of iron recycling genes. While HIF-1 has been shown to regulate TfR1 and HO-1 in other contexts, and has been anticipated to have a role in erythrophagocytosis,[9-11] we show here that macrophage-specific deletion of HIF-1 does not affect iron recycling. Besides, while HIF-2 is essential for iron absorption by regulating DMT-1 and FPN in the enterocytes,[6,8] it is not essential for the regulation of these genes during erythrophagocytosis. Our findings that neither HIF-1 nor HIF-2 are necessary to regulate key iron recycling genes are important to avoid erroneous conclusions drawn from reviews.

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The online version of this article has a Supplementary Appendix.

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