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27 April 2024

UNIVERSITÀ DEGLI STUDI DI TORINO

Cell-specific regulation of Ferroportin transcription following experimentally-induced acute anemia in mice

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Abstract

Ferroportin (FPN), the sole characterized iron exporter, is mainly controlled by the peptide hormone hepcidin in response to iron, erythroid factors, hypoxia, and inflammation. In addition, intracellular iron level controls FPN translation by modulating the binding of Iron Responsive Proteins at the 5'UTR of FPN mRNA. Recently, Hypoxia Inducible Factor (HIF)2α has been shown to regulate FPN expression in intestinal cells.

Here we show that, during experimentally-induced acute anemia in mice, FPN is regulated at transcriptional level in a cell-specific manner. FPN mRNA level increases in duodenum and spleen macrophages, whereas it does not change in liver and is strongly down-regulated in erythroid precursors. These results were confirmed in Caco2 Raw264.7 and K562 cells treated with a hypoxic stimulus. Moreover, we found a differential expression of HIF1α and HIF2α in cells and tissues that might account for the specificity of FPN regulation.

Thus, hypoxia, by directly controlling hepcidin and its target FPN, orchestrates a complex regulatory network aimed at ensuring rapid iron recovery from the periphery and efficient iron utilization in the erythroid compartment.

Introduction

Iron homeostasis is maintained through the tight regulation of iron absorption by the intestine, iron recycling by macrophages and iron utilization mainly for hemoglobin synthesis [1]. Ferroportin (FPN) plays a crucial role in iron homeostasis being the only known iron exporter responsible for iron delivery from enterocytes and macrophages to the plasma, and for iron export from tissues stores [2; 3].

FPN expression is mainly regulated at a post-translational level by the peptide hormone hepcidin [4]. Following hepcidin binding to FPN, FPN is internalized, phosphorylated and subsequently degraded by lysosomes [5; 6]. When hepcidin concentration is low, the rate of FPN synthesis is greater than its degradation, resulting in higher amounts of FPN at the cell surface, thus increasing iron export. On the contrary, high hepcidin levels lead to FPN degradation and consequently to iron retention.

Moreover, FPN expression is regulated at a post-transcriptional level by intracellular iron concentration through the Iron Responsive Element (IRE) located in the 5'-untranslated region (UTR) of its mRNA [7; 8]. The importance of this element is highlighted by alterations in iron homeostasis seen in Pmc mice bearing a deletion in the FPN promoter region, which alters transcription start sites and eliminates the IRE in the 5'-UTR [9].

Recently, Taylor and co-authors demonstrated that FPN was induced under conditions of low iron by the transcription factor Hypoxia Inducible Factor (HIF)2α [10].

Finally, other papers demonstrated that FPN expression is also regulated at the mRNA level by hemoglobin/heme, hypoxia and inflammation [3; 11; 12; 13; 14; 15; 16].

The aim of this work is to analyse FPN regulation after acute anemia, a pathologic condition characterized by enhanced erythropoiesis, increased iron absorption and recycling, and rapid iron mobilization from hepatic stores.

Materials and Methods

Mice and treatment

Three month-old wild-type 129Sv male mice were used for experiments. Phlebotomy was performed by retro-orbital bleeding: a total of 1.5 ml of blood was extracted in two lots over 48 hours. Experiments were performed 1, 3 and 7 days after the first bleeding. Hematological parameters were determined by using an automatic analyzer (Dasit Sysmex XE-2100).

All experiments were approved by the animal studies committee of the University of Torino (Italy).

Determination of Tissue Iron Content

Mice were perfused via the aorta with PBS and non-heme tissue iron content was determined as previously reported [13].

Western blotting

Fifty µg of total protein extracts were separated on 8% SDS-PAGE and analyzed by Western blotting using antibodies against FPN [2] and actin (Santa Cruz Biothecnology Inc., Santa Cruz, California, USA).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from tissue and cell samples using Micro-to-Midi Total RNA Purification System (Invitrogen, San Giuliano Milanese, MI, Italy). For quantitative real-time polymerase chain reaction (qRT-PCR), 1 µg total RNA was transcribed into complementary DNA (cDNA) by M-MLV reverse transcriptase (Invitrogen) and random primers (Invitrogen). qRT-PCR was performed on a 7300 Real Time PCR System (Applied Biosystems, Monza, Italy) using Universal Probe Library System (Roche Diagnostics Corp., Milano, Italy) as previously described [13]. Primers and probes are listed in Supplementary Table S1.

Nuclear protein extraction and Western blotting

Cells treated with $CoCl_2$ were washed once in PBS and lysed in Buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) on ice for 15 minutes. Cells were lysed with a needle and then centrifuged for 20 seconds at 12000g. Supernatant was discarded, the pellet washed with PBS and resuspendend in Buffer B (20 mM Hepes pH7.9, 25% (v/v) Glycerol, 0.42 NaCl, 1.5 mM $MgCl_2$, 0.2 EDTA, 0.5 mM DTT, 0.5mM PMSF, 1% Protease Inhibitor (Roche) followed by rotation at 4°C for 30 minutes. After centrifugation at 12000g at 4°C, the supernatant containing nuclei was collected, and protein concentration was determined using the Bio-rad Assay (Biorad, München, Germany). Fifty µg of nuclear protein extracts were separated on 10% SDS-PAGE, and

analyzed by Western blotting using a mouse monoclonal anti-HIF1α antibody (NB100-105; Novus Biologicals), a rabbit polyclonal anti-HIF2α antibody (NB100-122SS; Novus Biologicals) and a mouse monoclonal anti-HIF1β antibody (NB100-124; Novus Biologicals).

Spleen cell separation

Macrophages and erythroid precursor cells were isolated from spleen cell suspensions using CD11b and TER119 ferromagnetic microbeads respectively, according to the manufacturer's instructions (Miltenyi Biotec, Calderara di Reno, BO, Italy).

Cell culture and treatments

Raw264.7 cells (ATCC: TIB-71), K562 cells (ATCC: CCL-243™) and Caco2-cells (ATCC: HTB-37™) were used for in vitro experiments. Hypoxia on Raw Raw264.7 cells and K562 cells was chemically induced using cobalt chloride ($CoCl_2$; Sigma-Aldrich, Milano, Italy). After two hours of starvation, Raw264.7 and K562 cells were treated with 100-500 µM of $CoCl_2$ in culture medium without FCS, for different times. To block transcription, Actinomycin D (Sigma-Aldrich) was used together with $CoCl_2$ treatment at different concentration (from 10 to 100 µg/mL) for 24 hours.

For Caco-2 cells the hypoxia treatment was achieved by incubation for 6, 24, 48 hours in a hypoxia chamber (Billups-Rothenberg Inc., Del Mar CA) containing 1% oxygen., 5% carbon dioxide and 94% nitrogen gas.

Results

FPN is regulated in a tissue specific manner following phlebotomy

To gain an insight into the physiological response of FPN to experimentally-induced acute anemia in mice, we chose the bleeding model. A total of 1.5 ml of blood was extracted by retro-orbital puncture (in two lots) over 48 hours. Experiments were performed 1, 3 and 7 days after the first bleeding. Red blood cells (RBCs), hemoglobin (HGB), and hematocrit (HCT) values were reduced by approximately 50% in phlebotomized mice 3 days after phlebotomy, while reticulocytes were increased by approximately 60%. Expansion of the erythroid compartment was confirmed by the two-fold increase in spleen/body weight ratio 3 days after phlebotomy. Iron stores decreased significantly in the liver of phlebotomized mice (Figure 1A). As expected, qRT-PCR analysis revealed a 80% decrease in hepcidin mRNA level in livers of phlebotomized mice (Figure 1B). Accordingly, FPN expression was strongly induced in liver and duodenum whereas we could not detect such induction in spleen extracts. However, the increase in FPN expression was evident in macrophages isolated from the spleen of phlebotomyzed mice (Figure 1C-F). These results were expected as it is well-established that anemia negatively regulates hepcidin, thus increasing the stability of FPN at the cell plasma membrane [6; 17].

More interesting were the results obtained from the analysis of FPN mRNA expression following phlebotomy. We found that FPN mRNA level was strongly increased in duodenum and spleen macrophages, whereas its level did not change in the liver and was significantly down-regulated in TER119+-spleen erythroid precursors (Figure 2).

Thus, both changes in mRNA expression and protein stabilization, due to hepcidin down-regulation, contribute to regulate FPN expression following phlebotomy.

Hypoxia regulates FPN transcription in a cell-specific manner

We hypothesized that bleeding-induced hypoxia might be responsible for the tissue-specific regulation of FPN expression. HIF2α has already been shown to regulate FPN transcription in duodenum [10; 18]. Accordingly, FPN was induced in the intestinal cell line Caco2 following hypoxia (Figure 3A; Figure S1A). Similar results were obtained on Caco2 cells using a known and widely accepted hypoxia-mimicking agent, cobalt chloride (not shown). To assess whether hypoxia was also responsible for FPN up- and down-regulation in macrophages and erythroid progenitors respectively, FPN expression was analyzed in Raw264.7 (macrophage cell line) and K562 (erythroid-like cell line) cells treated for 24 hours with cobalt chloride ($CoCl_2$) (Figure S1B-C). After $CoCl_2$ incubation, FPN mRNA was up-regulated in Raw264.7 cells and down-regulated in K562 cells (Figure 3B-C). The positive effect on FPN expression was at a transcriptional level, as actynomicin D blocks FPN mRNA induction in Raw264.7 cells in a dose-dependent manner (Figure 3D).

HIF subunits are differentially expressed in cells and tissues

Data reported in the previous paragraph demonstrated that hypoxia may both positively and negatively control FPN transcription. To further investigate this issue we analyzed expression of the different HIF subunits, HIF1α, HIF2α and HIF3α, as it has already been reported that differential expression of these proteins may be responsible for a distinct pattern of cellular expression [19]. The most abundant isoform in Raw264.7 cells was HIF1α whereas in K562 and Caco2 cells was HIF2α; HIF3α was expressed at negligible levels in each cell type (Figure 4). The same pattern of HIF subunits expression was also seen in vivo: the most abundant isoform in

spleen macrophages was HIF1α whereas in the duodednum and erythroid cells was HIF2α (not shown).

Discussion

Here we demonstrated a tissue-specific regulation of FPN mRNA expression following phlebotomy: FPN mRNA increases in tissues that have to supply iron for erythropoiesis and decreases in erythroid progenitors that actively differentiate to recover anemia.

Up-regulation of FPN expression at mRNA level has already been reported in duodenums isolated from mice exposed to hypoxia or fed on iron-deficient diet [3]. Treatment of CaCo2 cells with iron reduces FPN transcriptional rates while desferrioxamine treatment results in the opposite effect [16]. Moreover, iron loading of human alveolar macrophages [20] and murine J774 macrophages [21] results in increased FPN mRNA. Finally, FPN mRNA levels increase in bone marrow-derived macrophages following erythrophagocytosis and heme treatment, [11; 22; 23] as well as in Raw264.7 cells after hemoglobin supplementation [13]. Inflammatory stimuli, such as LPS [12], TNFα [24] and turpentine oil [15] can also regulate FPN mRNA levels, both in vivo and in vitro. In addition, it has been reported that high-altitude exposure is associated with an increase in skeletal muscle FPN mRNA levels in humans [14].

Our data indicate that hypoxia consequent to bleeding may be responsible for the tissue-specific regulation of FPN expression as we were able to reproduce in vitro the same pattern of FPN expression observed in vivo by treating Caco2, Raw264.7 and K562 cells with an hypoxic stimulus. However we cannot rule out the possibility that other stimuli induced by bleeding may concur to regulate FPN expression. Among these, erythropoietin (EPO) has been shown to induce

FPN expression in Caco2 cells [25] and in humans erythropoietin administration affects FPN expression in muscle [26] and in peritoneal exudates macrophages [27]. However, the administration of recombinant human EPO to mice did not affect FPN expression in the duodenum [28]. Whether EPO directly affect FPN promoter has not yet been investigated. Moreover, it has been reported that FPN promoter could be regulated by putative erythroid-specific transcription factors, like GATA and KLF. Accordingly, FPN expression decreased both at the mRNA and protein levels during estradiol-induced erythroid differentiation of G1E-ER4 cells [29]. Our data suggest that HIF may represent an additional transcription factor involved in the regulation of FPN expression at mRNA level in conditions of acute anemia.

We showed that HIFα subunits are differentially expressed in mouse tissues, HIF1α being mainly expressed in macrophages and HIF2α in duodenal cells and erythroid progenitors. HIF-1α and HIF-2α are characterized by similar structures and both recognize the same DNA sequence. However, differences in their N-terminal activation domain and the requirement of specific transcriptional cofactors confer them specificity in the regulation of gene expression [30]. It has already been demonstrated that HIF1α and HIF2α subunits can exhibit opposite roles in the regulation of a single target. As an example, in endothelial cells HIF-1α diminishes the expression of interleukin-8 (IL-8) by inhibition of the Nrf2 transcription factor, while in the same cells HIF-2α increases the expression of IL-8 in an Nrf2-independent way. Additionally, HIF-1 decreased, whereas HIF-2 increased, c-Myc expression [30]. Moreover, the complexity of the HIF regulatory system is further increased by the possibility of a single HIFα subunit to induce opposite effects on the same target gene in different cells. Indeed, Fenglan Lou et al. demonstrated that HIF-2α enhances telomerase reverse transcriptase (hTERT) gene expression in renal cell carcinoma (RCC) cell lines, whereas it represses the hTERT transcription in glioma cells [31].

Based on these observations, we speculate that the differential expression of HIF-1α and HIF-2α, together with tissue-specific co-factors, is responsible for the cell-specific regulation of FPN transcription.

Hypoxia is a main regulator of iron homeostasis as it strongly down-regulates hepcidin [32], likely through a mechanism involving both HIF1α and HIF2α [33]. It is intriguing that the same stimulus may both inhibit hepcidin expression and induce its target protein, FPN, in duodenum and macrophages, thus ensuring an adequate iron supply for erythropoiesis. On the other hand, in erythroid precursors hypoxia down-regulates FPN, as shown here, and induces transferrin receptor thus allowing efficient iron utilization [34].

Thus, hypoxia orchestrates a complex regulatory network aimed at ensuring a rapid mobilization of iron from duodenum and macrophages and, on the other hand, at limiting the loss of iron, which is required for hemoglobin synthesis in erythroid precursors (Figure 5).

Authorship

D.C. performed experiments, analyzed results and wrote the paper; V.F. and S.M. performed some experiments; L.S. supervised experimental work; F.A. designed the research and reviewed the paper; E.T. designed the research, analyzed results, and wrote the paper.

The authors reported no potential conflicts of interest.

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Legends for Figures

Figure 1. Regulation of FPN expression following phlemotomy.

(**A**) Phlemotomy model. Hematological parameters, liver iron and spleen/body weight ratio in untreated and phlebotomized mice 1, 3 and 7 days after the first bleeding. Data represent mean ± SEM; n=10 for each experimental point. * = P<0.05; ** = P<0.01; *** = P<0.001. (**B**) qRT-PCR analysis of hepcidin expression in the liver of untreated and phlebotomized mice, 3 days after the first bleeding. Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM; n=5; *** = P<0.001. Results shown are representative of 3 independent experiments. (**C-F**) Western blots of FPN expression in liver, duodenum, spleen and CD11b+ cells isolated from the spleen of untreated and phlebotomized mice, 3 days after the first bleeding. A representative experiment for each tissue is shown. Band intensities were measured by densitometry and normalyzed to actin expression. Data represent mean ± SEM; n=3 for each experimental point. ** = P<0.01; *** = P<0.001.

Figure 2. Cell-specific transcriptional regulation of FPN after phlebotomy. qRT-PCR analysis of FPN expression in liver (**A**) duodenum (**B**) spleen CD11b+ cells (**C**) and spleen TER119+ cells (**D**) of untreated and phlebotomized mice, 3 days after the first bleeding. Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM; n=3; * = P<0.05. Results shown are representative of 3 independent experiments.

Figure 3. Hypoxia controls FPN transcription. (**A**) qRT-PCR analysis of FPN mRNA levels in Caco-2 cells following 6, 24 and 48 hours of hypoxia (1% oxygen). (**B**) qRT-PCR analysis of FPN mRNA levels in RAW 264.7 and K562 cells treated for 8, 12, 18 or 24 hours with 100 μM CoCl$_2$. (**D**) qRT-PCR analysis of FPN expression in RAW264.7 treated for 24 hours with 100 μM CoCl$_2$ and increasing amounts of actinomycin D. Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM; n=3; * = P<0.05; *** = P<0.001. Results shown are representative of 3 independent experiments.

Figure 4. Differential expression of HIF α subunits. qRT-PCR analysis of HIF1α, HIF2α and HIF3α expression in RAW264.7 (**A**), K562 (**B**) and Caco-2 (**C**) cells. Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM; n=3 for each experimental point; ** = P<0.01; *** = P<0.001. Results shown are representative of 3 independent experiments.

Figure 5. Cell-specific transcriptional regulation of FPN is mediated by different HIF subunits. We hypothesized that hypoxia may stabilize different HIFα subunits in different cell types. Stabilization of HIF1α in macrophages, in association with the constitutive HIF1β isoform, activates the transcription of FPN mRNA. HIF2α plays a role in the transcriptional activation of FPN mRNA in the duodenum [10]. In this manner hypoxia contributes at the transcriptional level to increase FPN expression in duodenum and macrophages, thus allowing increased iron absorption and mobilization to support erythropoiesis.

Data reported here suggested that HIF2α could inhibit FPN transcription in erythroid progenitors. The down-regulation of FPN protein may be important to increase iron availability for heme synthesis in erythroid progenitors.

We hypothesized that the association of HIF2α with cell-specific transcriptional activators or repressors could account for the differential role of HIF2α in modulating the transcription of FPN in duodenum and erythroid precursors.

A

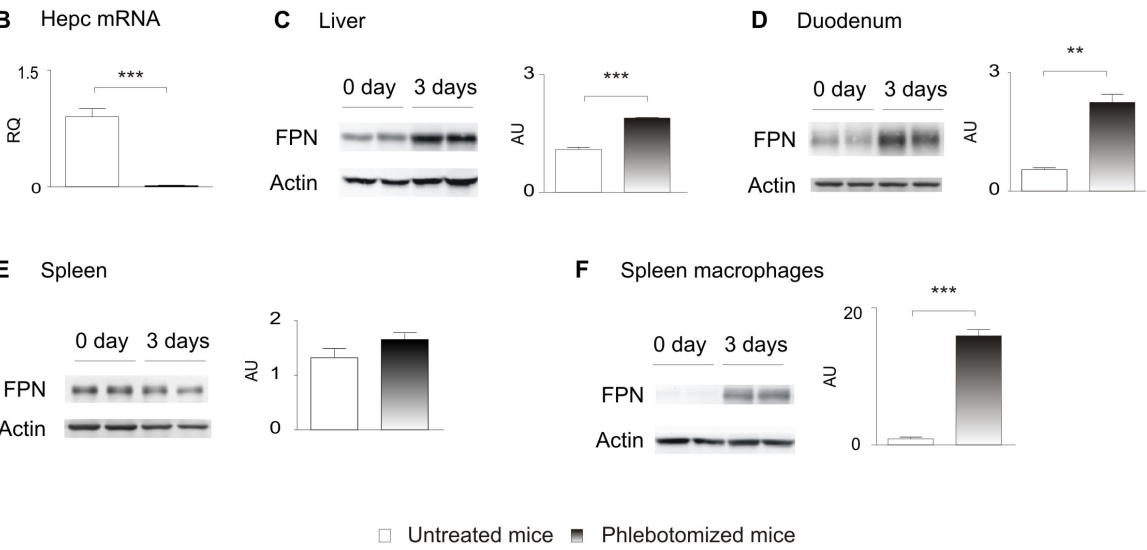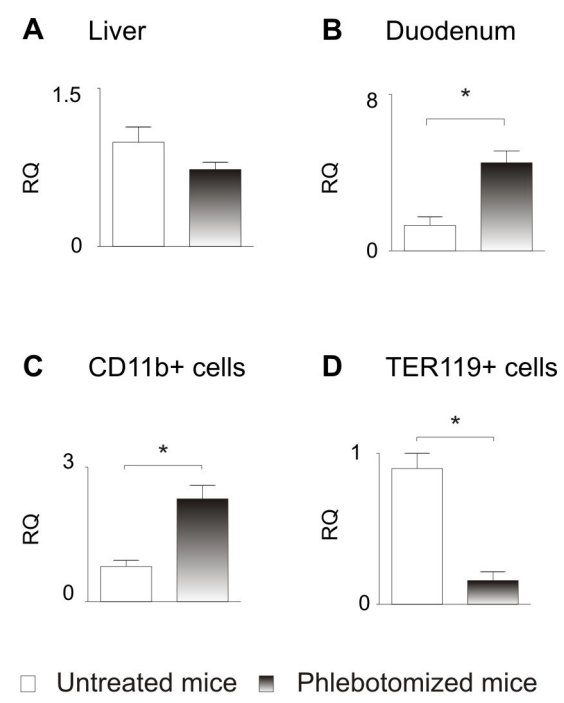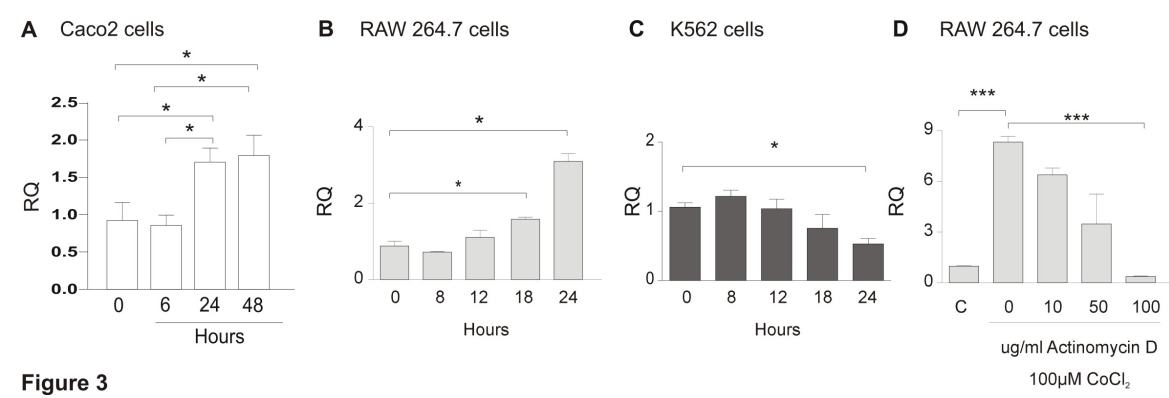|	0 days	1 days	3 days	7 days
HCT (%)	43,51 ± 0,59 (n=12)	33,95 ± 1,09 *** (n=11)	27,39 ± 1,60 *** (n=8)	39,0 ± 0,60 ** (n=8)
RBC (10^12/L)	9,14 ± 0,15 (n=12)	7,03 ± 0,25 *** (n=11)	5,03 ± 0,33 *** (n=8)	7,15 ± 0,28 *** (n=8)
HGB (g/dL)	13,58 ± 0,20 (n=12)	10,18 ± 0,35 *** (n=11)	7,64 ± 0,48 *** (n=8)	11,37 ± 0,13 *** (n=8)
RET (%)	3,45 ± 0,48 (n=12)	4,51 ± 0,44 (n=11)	7,44 ± 0,61 *** (n=8)	8,35 ± 1,06 *** (n=8)
MCV (fL)	47,48 ± 0,23 (n=12)	48,28 ± 0,40 (n=11)	54,79 ± 0,78 *** (n=8)	54,45 ± 0,97 *** (n=8)
Liver Iron (µg/g)	292.8 ± 19.90 (n=8)	\	236.9 ± 13.12 * (n=8)	189.0 ± 27.42 ** (n=5)
Splenic weight ratio	5.65 ± 0.43 (n=6)	\	11.56 ± 0.70 *** (n=3)	

B Hepc mRNA

C Liver

D Duodenum

E Spleen

F Spleen macrophages

☐ Untreated mice ■ Phlebotomized mice

Figure 1

A Liver

B Duodenum

C CD11b+ cells

D TER119+ cells

□ Untreated mice ■ Phlebotomized mice

Figure 2

A Caco2 cells   **B** RAW 264.7 cells   **C** K562 cells   **D** RAW 264.7 cells

Figure 3

A RAW 264.7 cells **B** K562 cells **C** Caco2 cells

Figure 4

Figure 5