PALACKÝ UNIVERSITY OLOMOUC

Faculty of Medicine and Dentistry

Department of Biology

NEW INSIGHTS INTO ERYTHROPOIESIS AND IRON METABOLISM – LESSONS LEARNED FROM INHERITED DEFECTS OF RED BLOOD CELL PRODUCTION

Habilitation thesis

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This work is dedicated to my parents Greta and Walter and to my grandmother Anna, who have raised me up with love and diligence and have always supported me.

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Last but not least, I want to thank my husband Marian and my son Matus for just being here for me.

Hereby I declare that the presented habilitation thesis is cumulative, based on my previous research.

CONTENT

1. Introduction

1.1 Erythropoiesis

Erythropoiesis is a continual process of red blood cell/erythrocyte production. Erythrocytes lack nuclei and are rich in an iron-containing molecule, hemoglobin that is essential for oxygen binding and delivery to the body cells. The level of tissue oxygenation influences erythrocyte production in a feedback loop. Erythropoiesis is tightly coupled with hemoglobin production which in turn depends on three biological processes: synthesis of globins, synthesis of the heme precursor (protoporphyrin IX), and iron uptake (reviewed in Koury et al., 2002) (Fig. 1). These processes need to be strictly regulated and coordinated as any disbalance may lead to different pathological conditions; some of them will be discussed in this habilitation thesis.

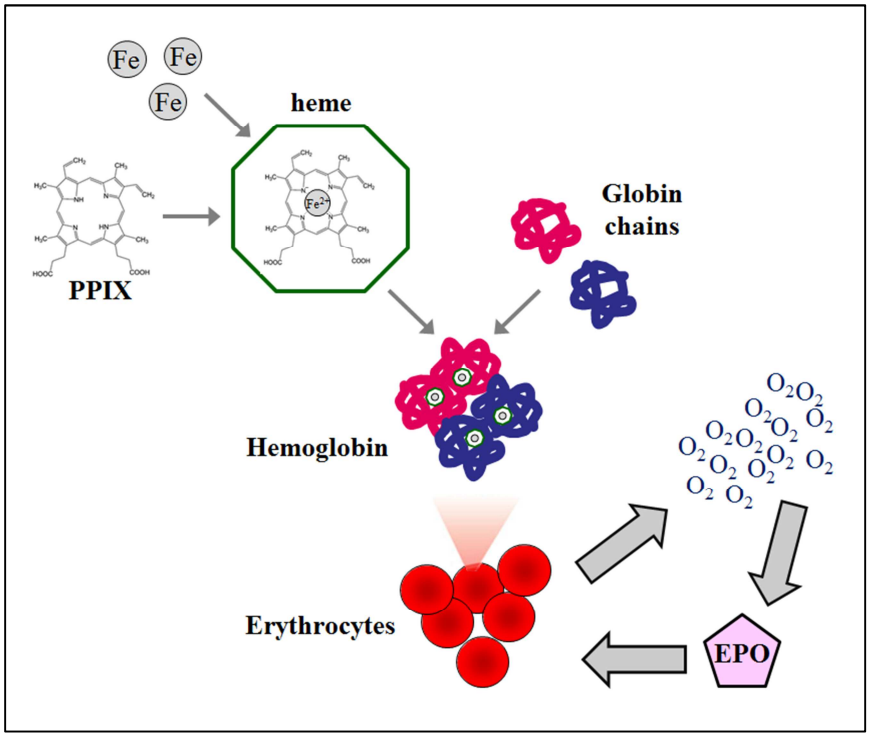

Figure 1: Erythrocytes, hemoglobin, and erythropoietin production. Formation of erythrocytes is associated with hemoglobin synthesis, which is dependent on iron uptake (Fe), heme synthesis (the last step is the incorporation of Fe into protoporphyrin IX – PPIX), and production of globin polypeptides. The size of red blood cell mass influences tissue oxygenation (O_2), which in a feedback loop via erythropoietin (EPO) regulates production of erythrocytes in the bone marrow.

During mammalian ontogeny erythropoiesis occurs in successive but overlapping waves that take place in the yolk sack, fetal liver/spleen, and bone marrow. The yolk sack erythropoiesis, called primitive erythropoiesis, is characterized by production of large nucleated erythroblasts, which synthesize embryonic hemoglobins. The first definitive erythroid progenitors emerge in the yolk sac and in the aorta-gonad-mesonephros (AGM) region, but the main site of definitive erythropoiesis in the embryo is the fetal liver. Fetal liver definitive erythroblasts mature to enucleated erythrocytes. Around the birth, bone marrow becomes the principal site of adult erythropoiesis (Orkin and Zon, 2002). Hematopoietic stem cell (HSC) differentiate first to multipotent progenitor CFU-GEMM (colony-forming unit granulocytic, erythroid, megakaryocyte, macrophage) and then through unipotent lineage-committed progenitors, early erythroid progenitor BFU-E (burst-forming units-erythroid) and late erythroid progenitor CFU-E (colony-forming units-erythroid) to morphologically recognizable erythroid precursors (proerythroblasts and erythroblast) (Gregory and Eaves, 1978). Erythroid precursors then undergo significant biochemical and morphological changes including increase in iron uptake and hemoglobin synthesis, cell shrinking, chromatin condensation, and enucleation and finally produce reticulocytes and mature erythrocytes which are released into the blood stream (Fig. 2).

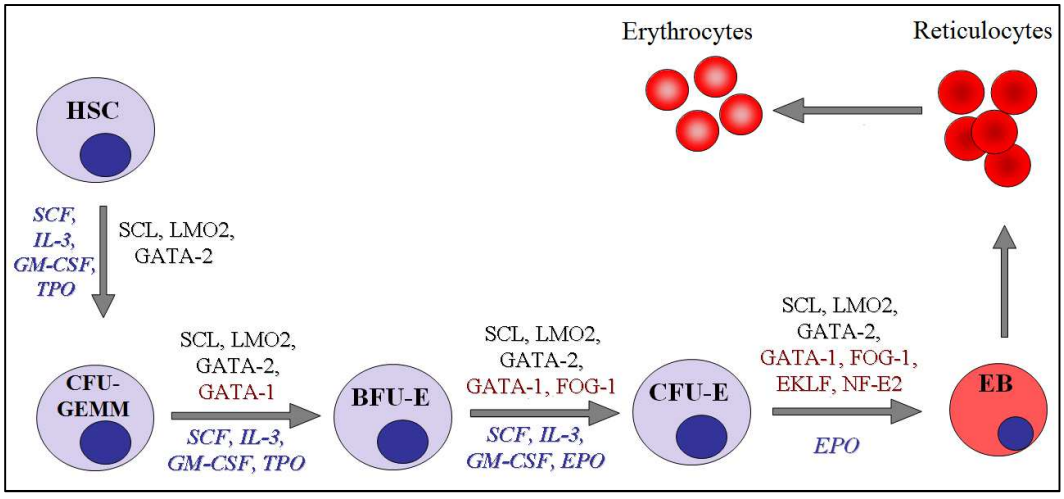

Figure 2: Erythroid differentiation of hematopoietic stem cell (HSC) through progenitor (CFU-GEMM, CFU-E, BFU-E) and precursors stages (EB - erythroblast). The dependence of different stages on transcription factors and growth factors is also depicted. The progenitors can be isolated from peripheral blood and used for the *in vitro* colony assay: in semisolid media supplemented with cytokines hematopoietic progenitors proliferate, differentiate, and form corresponding colonies (Migliaccio et al., 1988). The number of colonies reflects the hematopoietic activity in the bone marrow. This test enables evaluation of the effect of different drugs or molecules, for example the test of sensitivity of erythroid progenitors to EPO, which is used in differential diagnostics of polycythemia states.

1.1.1 Regulation of erythropoiesis

Erythroid differentiation, maturation, and survival are tightly regulated and controlled by a number of transcription factors and growth factors. While transcription factors SCL/TAL-1 (stem cell leukemia/T-cell acute leukemia) (Shivdasani et al., 1995), LMO2/rbtn2 (Warren et al., 1994), and GATA-2 (Tsai and Orkin, 1997) are required for hematopoiesis in general and their knock-out in mouse is embryonic lethal (due to the absence/severe suppression of primitive and definitive hematopoiesis), others are essential only for erythropoiesis/megakaryopoiesis, like GATA-1 (Fujiwara et al., 1996), FOG-1 (friend of GATA-1) (Tsang et al., 1998), EKLF (erythroid Krüppel-like factor) (Perkins et al., 1996), and p45NF-E2 (nuclear factor erythroid-2) (Shivdasani and Orkin, 1995) (Fig. 2).

Among these transcription factors GATA-1 is one the most important for the terminal erythroid maturation; it controls the expression of a) genes involved in hemoglobin production (i.e. α- and β-globin genes, some heme biosynthetic pathway genes); b) gene coding for EPO receptor (EPOR); c) genes encoding transcription factors MafK and p45NF-E2 (Weiss et al., 1994). Besides erythroid differentiation, GATA1 is also involved in the regulation of cell cycle progression and survival.

The cytokines which positively influence erythropoiesis include: stem cell factor (SCF), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), thrombopoietin (TPO), and erythropoietin (EPO) (Fig. 2). However, only SCF and EPO are absolutely required for erythroid development. Their effect on erythropoiesis is mainly sequential; SCF is essential for proliferation and survival of early and late stages of erythroid progenitors (Muta et al., 1995), while EPO is critical for later stages of erythroid progenitors and maturating erythroblasts (Wu et al., 1995). Nevertheless, SCF and EPO were shown to act also synergistically to support erythroid cell production (Wu et al., 1997; Sui et al., 1998).

Erythropoiesis needs to be regulated also negatively to avoid excessive production of erythrocytes. This involves induction of apoptosis via death receptors and their ligands: Fas/FasL, TNFR/TNF-α or TRAILR/TRAIL (Ulich et al., 1990; Schneider et al., 1999; De Maria et al., 1999a). For example, in low EPO conditions mature erythroblasts expressing FasL induce apoptosis of immature erythroblasts expressing Fas (De Maria et al., 1999a).

Induction of apoptosis is associated with caspases activation. Caspases subsequently cleave transcription factors, GATA1 and SCL/TAL1, crucial for erythropoiesis (De Maria et al., 1999b; Zeuner et al., 2003). A number of inflammatory cytokines also exert their inhibitory effect on erythropoiesis via death receptors; interferon γ (INF γ) was shown to induce the expression of Fas (Dai et al., 1998) and TNF-α directly binds to TNFR expressed on immature erythroblasts. The action of transforming growth factor β (TGF-β) differs from the previous two as it accelerates differentiation and blocks proliferation of erythroid progenitors (Zermati et al., 2000).

1.1.2 EPO/EPOR signaling

The principal hormone that regulates erythrocyte production is EPO. Before birth, EPO is predominantly produced in the fetal liver by hepatocytes (Dame et al., 1998). At birth, EPO production switches from the fetal liver to the kidney, where peritubular interstitial fibroblasts produce EPO (Maxwell et al., 1993) in response to tissue oxygenation. EPO exerts its effects on erythroid cells by binding to specific receptor, erythropoietin receptor (EPOR), expressed on their surface. The EPO/EPOR interaction leads to homodimerization of EPOR and signal transduction that results in stimulation of mitogenicity and erythroid differentiation/maturation, and prevention of apoptosis of erythroid cells (Remy et al., 1999) (Fig. 3).

Although EPO and EPOR knock-out is embryonic lethal in mouse, due to severe anemia; detailed analysis revealed that EPO/EPOR signaling is only essential for survival and terminal erythroid differentiation but dispensable for the erythroid lineage specification (Wu et al., 1995; Lin et al., 1996). The first cells in the erythroid lineage that become EPO dependent are CFU-E progenitors (Wu et al., 1995). These cells also express the highest levels of EPOR; beyond this stage EPOR expression progressively declines as the cells proceed in differentiation.

EPOR belongs to a family of cytokine receptors that do not possess intrinsic kinase activity and depend on the activity of associated kinase; in case of EPOR the Janus tyrosine kinase-2 (JAK2) (Ihle, 1995). EPO binding to EPOR leads to autophosphorylation of JAK2. Activated JAK2 then phosphorylates EPOR tyrosine residues, which serve as

docking sites for binding of intracellular proteins with src homology domain (SH2); these proteins are phosphorylated and activated by JAK2 upon binding to EPOR. The best known cascades stimulated by EPO include anti-apoptotic phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Damen et al., 1993), mitogenic RAS/MAPK pathway (Miura et al., 1994), and signal transducer and activator of transcription-5 (STAT5) (Sawyer and Penta, 1996) (Fig. 3).

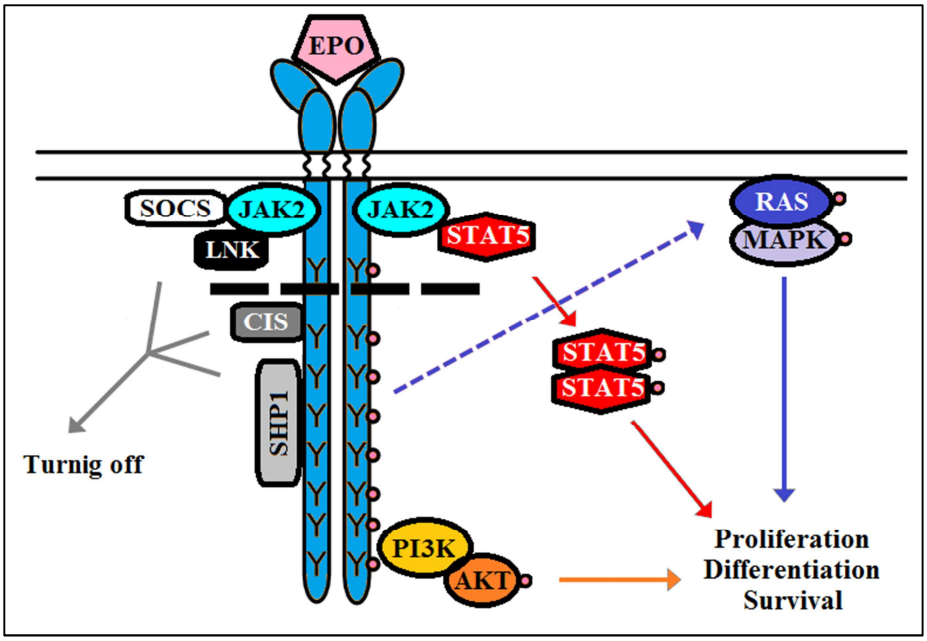

Figure 3: EPO/EPOR signaling. EPO binding to EPOR results in receptor homodimerization and autophosphorylation of the receptor-associated JAK2. Activated JAK2 phosphorylates the key tyrosine residues (Y) on the cytoplasmic region of EPOR and activates STAT5, PI3K/AKT, and RAS/MAPK downstream signaling responsible for proliferation, differentiation, and survival of erythroid cells (right side). Negative regulation of EPO/EPOR signaling by SOCS, CIS, SHP1, and LNK is depicted on the left side (for details see the main text). Thick dashed line indicates the truncation of EPOR due to EPOR mutations associated with primary familial congenital polycythemia – PFCP, leading to the loss of negative regulatory regions.

The JAK2/STAT5 signaling plays a non-redundant, essential role in EPO/EPOR-mediated regulation of erythropoiesis by supporting survival and differentiation of erythroid cells. The main mediators of the anti-apoptotic effect are members of the Bcl-2 family, namely $BclX_L$ and Mcl-1 (Socolovsky et al., 1999; Gregoli and Bondurant, 1997; Rhodes et al., 2005). EPO/EPOR also stimulates Lyn (Ingley et al., 2005) and Btk (Schmidt et al., 2004) kinases. Lyn is proposed to function via erythroid cell-intrinsic mechanisms involving activation of STAT5 and $BclX_L$ to promote progenitor cell expansion and to support development of subsequent late erythroblast stages (Karur et al., 2006).

The cytoplasmic domain of EPOR contains besides positive regulatory regions (Fig. 3, right side) also negative regulatory regions (Fig. 3, left side). These regions are necessary for down-regulation and termination of the transduced signal (D'Andrea et al., 1991). Hematopoietic cell phosphatase (HCP, also known as SHP-1) binds to EPOR to dephosphorylate the tyrosine residues phosphorylated by JAK2 (Klingmuller et al., 1995). Other negative regulators belong to a family of suppressors of cytokine signaling (SOCS) and act in a negative feedback loop to deactivate the EPOR/JAK2 complex (SOCS 1, SOCS 3) or interfere for STAT5 binding to EPOR (CIS) (Krebs a Hilton, 2001). Another important negative regulator is the lymphocyte adaptor protein LNK (also known as SH2B3). LNK itself becomes tyrosine-phosphorylated shortly following EPO stimulation and via its SH2 domain inhibits EPO-induced JAK2 activation and EPOR phosphorylation leading to attenuation of STAT5, AKT, and PI3K pathways (Tong et al., 2005).

1.1.3 Hypoxia inducible signaling

The production of EPO is mainly controlled at the transcriptional level. EPO expression is induced in response to inadequate oxygen supply by hypoxia inducible transcription factors (HIFs, predominantly HIF2) (Wang and Semenza, 1993; Warnecke et al., 2004) (Fig. 4). HIFs are heterodimers composed of an inducible alpha subunit (HIFα) and a constitutively-expressed beta subunit (HIFβ, also known as aryl hydrocarbon receptor nuclear translocator, ARNT).

In normoxic conditions, HIFα is hydroxylated at conserved proline residues by HIF prolyl-hydroxylases (PHD1-3), allowing HIFα recognition and ubiquitination by an E3 ubiquitin ligase complex, which contains von Hippel Lindau protein (pVHL) as the recognition subunit. This ubiquitination results in rapid proteasomal degradation of HIFα. In hypoxia, PHDs are inactive, since they require oxygen as a co-substrate. Besides oxygen also iron is needed for PHDs, as it acts as a catalytic cofactor. Non-hydroxylated HIFα is not recognized by pVHL, leading to its stabilization, heterodimerization with HIFβ, and nuclear translocation. Upon binding to hypoxia response elements (HRE) within the promotor of target genes, HIF transcription factors induce gene expression (reviewed in Semenza, 2009) .

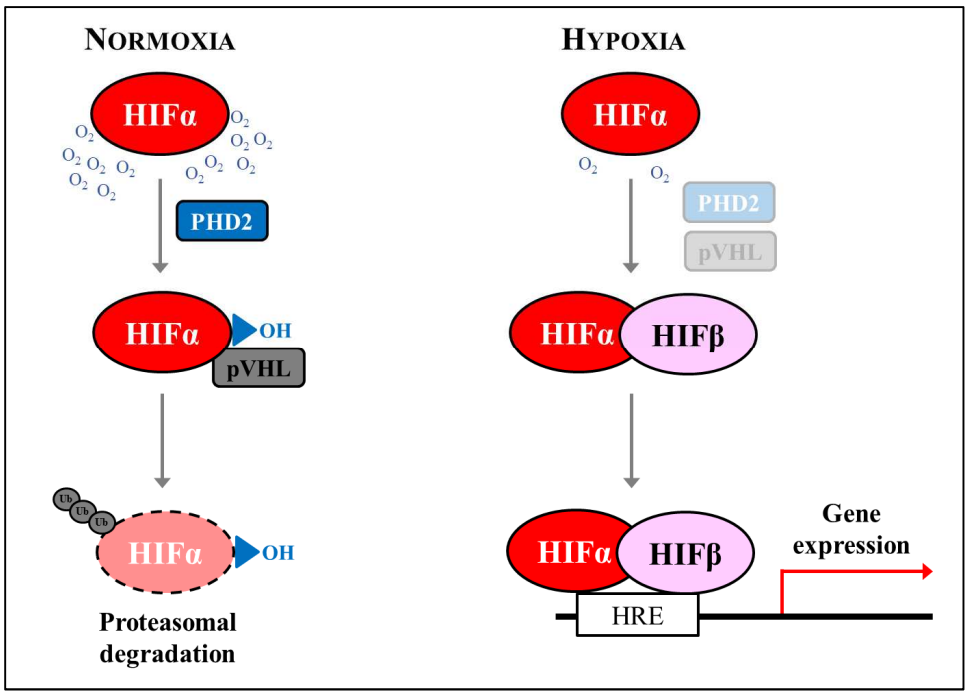

Figure 4: Hypoxia inducible signaling. Normoxia: HIF-α is hydroxylated by PHDs on proline residues leading to its recognition by pVHL and subsequent proteasomal degradation. Hypoxia: HIF-α is stabilized and forms heterodimers with HIF-β. This complex binds to the hypoxia-responsive elements (HRE) and induces gene expression.

The direct transcriptional HIF targets include, besides *EPO*, genes encoding proteins involved in a) energy metabolism: glucose transporter-1 (*SLC2A1*), hexokinase-1 and 2 (*HK1*, *HK2*); b) angiogenesis: vascular endothelial growth factor (*VEGF*), vascular endothelial growth factor receptor-1 (*Flt-1*); c) iron metabolism: transferrin (*Tf*), transferrin receptor-1 (*TfR1*), intestinal form of divalent metal transporter 1 (*DMT1*, containing the iron responsive element + IRE), heme oxygenase-1 (*HO-1*), and d) apoptosis: *Bnip3*.

1.1.4 Hemoglobin

Hemoglobin (Hb) is a complex iron containing protein responsible for oxygen binding that makes up about 96% of red blood cell dry content. Hemoglobin is composed of four subunits; each subunit has a protein part – **globin chain** and a non-protein part – **heme** consisting of a protoporphyrin ring and a central iron atom (Fe^{2+}) (Fig. 1). Different types of hemoglobin are produced sequentially during ontogenesis in man (Fig. 5); each comprised of one pair of α-like globin chains produced from genes located in the α-globin

cluster on chromosome 16 and the second pair of β-like globin chains produced from genes located in the β-globin cluster on chromosome 11 (reviewed in Sankaran et al., 2010).

Two main types of hemoglobin are synthesized during the postnatal period: HbA (representing about 98% of total hemoglobin and consisting of two α-globin chains and two β-globin chains) and HbA2 (representing about 1-3% of total hemoglobin and consisting of two α-globin chains and two δ-globin chains). Fetal hemoglobin (HbF, consisting of two α-globin chains and two γ-globin chains) is also present in a few percentages of mature erythrocytes in healthy individuals and represents a minor component of total hemoglobin (up to 1%).

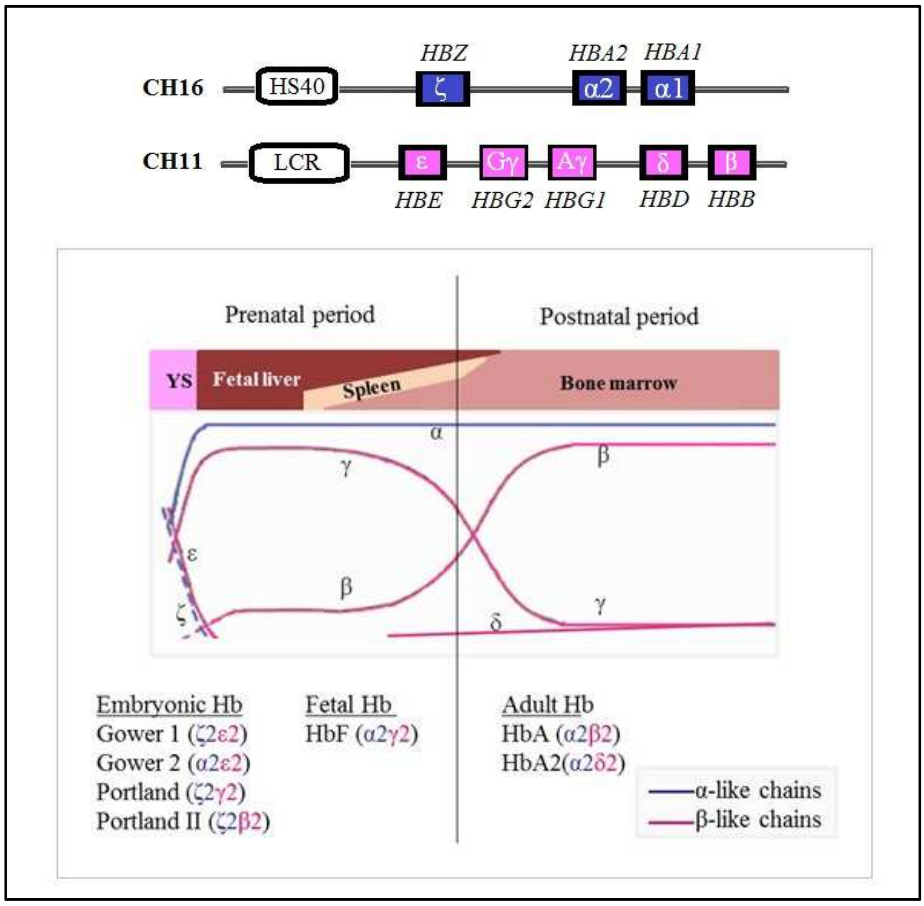

Figure 5: Hemoglobin switching in man. Different types of hemoglobin (Hb) are produced during different stages of ontogenesis, *i.e.*embryonic, fetal, and adult. This coincides with the transition of the site of erythropoiesis from the yolk sac to the fetal liver/spleen, and bone marrow. Each Hb molecule is comprised of one pair of α-like globin chains produced from genes located in the α-globin cluster on chromosome 16 and the second pair of β-like globin chains produced from genes located in the β-globin cluster on chromosome 11. The α-globin cluster contains three functional genes: *HBZ* coding for embryonic chain ζ and *HBA1* and *HBA2* coding for α1 and α2 chains, which are expressed since embryonic stage. The β-globin cluster is composed of five functional β-globin genes: *HBE* coding for embryonic ε chain; *HBG1* and *HBG2* coding for embryonic/fetal Aγ and Gγ chains; *HBD* and *HBB* coding for adult δ and β chains, respectively. HS40 (hypersensitive site 40) and LCR (locus control region) are regions regulating globin gene expression.

1.1.5 Regulation of globin synthesis and heme degradation

The expression of globin genes is erythroid specific and regulated at transcriptional and translational level; both levels are significantly influenced by the availability of iron, particularly heme. Heme is therefore not only the non-protein part of hemoglobin, but also an important signaling molecule influencing hemoglobin production.

Transcription of globin genes is stimulated by erythroid specific transcription factors GATA-1, EKLF (Hardison, 2001), and NF-E2. NF-E2 is a heterodimer composed of small Maf proteins (MafK) and erythroid specific subunit p45NF-E2 (Blank a Andrews, 1997). This heterodimer binds to Maf - recognition element (MARE) located in the regulatory region of β- and α-globin genes (Ney et al., 1990; Jarman et al., 1991). The NF-E2 activation complex is formed during erythroid differentiation when p45NF-E2 subunit replaces Bach1 repressor in the complex with MafK (Igarashi et al., 1998; Brand et al., 2004). This replacement is promoted by heme (Sun et al., 2004).

At the level of translation, heme influences the activity of **heme regulated inhibitory kinase – HRI**, which phosphorylates and inactivates the alpha subunit of the eukaryotic initiation factor – 2 (**eIF2α)** leading to repression of proteosynthesis (Chen et al., 1991) (Fig. 6). Heme binding to the insertion domain of HRI inhibits HRI activity thus allowing synthesis of globin chains proportional to heme levels (Chefalo et al., 1998; Rafie-Kolpin et al., 2000). On the other hand, globin production is inhibited by HRI in response to low heme levels to avoid production of excessive free globin chains (Uma et al., 2000).

Besides the role in the regulation of globin synthesis, heme also regulates its own synthesis and degradation (Karibian a London, 1965; Ponka et al., 1974).

Heme degradation is catalyzed by two isoenzymes: constitutively expressed heme oxygenase-2 (HO-2) and inducible heme oxygenase-1 (HO-1) (Poss a Tonegawa, 1997). The expression of HO-1 is stimulated by heme (Alam et al., 1989). In analogy to β–globin expression, heme induces displacement of Bach1 repressor from the MafK-occupied *HO-1* MARE sequences followed by binding of an activator Nrf2 (NF-E2 related factor 2), which leads to the initiation of *HO-1* transcription (Sun et al., 2004).

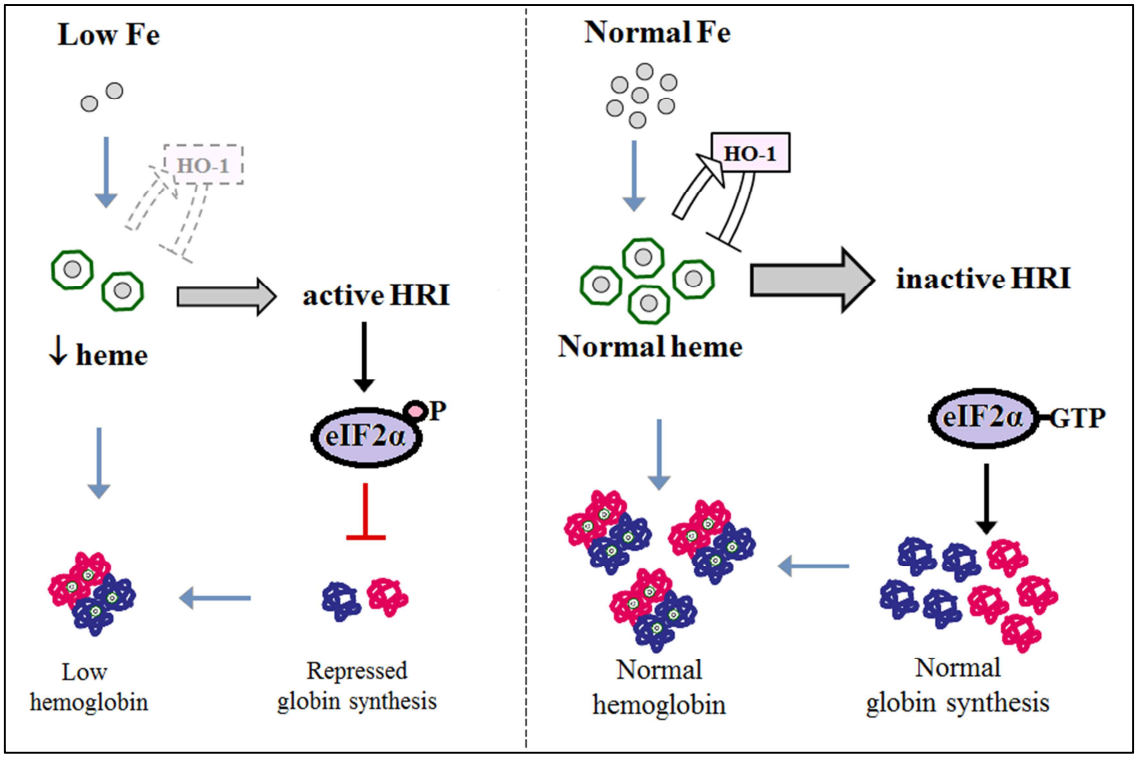

Figure 6. Regulation of globin synthesis by heme and HRI. When iron and heme are limiting, HRI kinase is active and phosphorylates eIF2α (depicted by P) leading to its inactivation and repression of globin synthesis (left side). In normal or iron replete conditions heme binds to the insertion domain of HRI and inhibits its kinase activity. Unphosphorylated eIF2α is active (bond to GTP) and initiates translation of globin chains proportional to heme levels (right side). In addition, heme also induces expression of HO-1 and thus regulates its own degradation. The control of the heme levels by HO-1 is important for normal process of erythropoiesis as discussed in Chapter 1.2.4.2.

1.2 Iron metabolism

Iron is an essential element indispensable for life. It is involved in many cellular and enzymatic processes including oxygen transport and storage, cell proliferation, and redox reactions. However, in excess, iron is toxic to the cell as it catalyzes the production of reactive oxygen radicals. Iron uptake, transport, storage, and utilization are therefore strictly regulated to meet the body's iron needs and to avoid its potential toxicity (Aisen et al., 2001).

The process of iron absorption, transport, utilization, and recycling is discussed in details in review articles which are presented as Attachments 7, 8, and 9 of this habilitation thesis. Therefore mainly new aspects of these processes and the importance of diagnostic parameters of iron status and erythropoiesis, which are discussed in **Chapter *2.2 Disordered iron homeostasis in congenital defects of erythropoiesis,*** are depicted here.

1.2.1 Duodenal iron absorption, ferritin, and transferrin

Iron uptake from the diet takes place mainly in the enterocytes of the proximal duodenum. Only about 10% of dietary iron (1–2 mg/day) is absorbed either in the form of heme or inorganic iron. Recent results suggested that also ferritin may represent an important dietary source of iron, but little is known about the mechanism of transport or its metabolism in the enterocytes (Theil et al., 2012).

Inorganic iron, reduced to ferrous state (Fe^{2+}) by duodenal ferrireductases (McKie et al., 2001; Gunshin et al., 2005a), is transported across the apical membrane of enterocytes by divalent metal transporter-1 (DMT1, also known as NRAMP2, DCT1, and SLC11A2) (Fleming et al., 1997; Gunshin et al., 1997; Gunshin et al., 2005b). The expresseion of this intestinal specific isoform of DMT1 (isoform I; +IRE/1A) is regulated by cellular iron levels (see detainls in Chapter *1.2.4.1*). Although heme iron is absorbed much more efficiently than inorganic iron, the identity of an intestinal heme transporter is still elusive. Once inside the enterocytes, iron is liberated from heme by the action of HO-1

(Boni et al., 1993). It is believed that the subsequent route of iron released from heme (and eventually from ferritin) is the same as the pathway of absorbed inorganic iron (Fig. 7).

Inside the enterocytes iron is either stored within the cells bound to ferritin (Harrison and Arosio, 1996) or exported to the circulation when iron becomes limiting. Iron retained within the enterocytes is rapidly lost from the body because of a short lifespan of these cells which are slough into the gut lumen.

Ferritin is the major intracellular iron storage protein of the body and its soluble form found in the plasma reflects body iron stores (with some exceptions such as cancer, inflammation, infection, etc.). Serum ferritin is therefore used as a diagnostic marker for iron deficiency and iron overload.

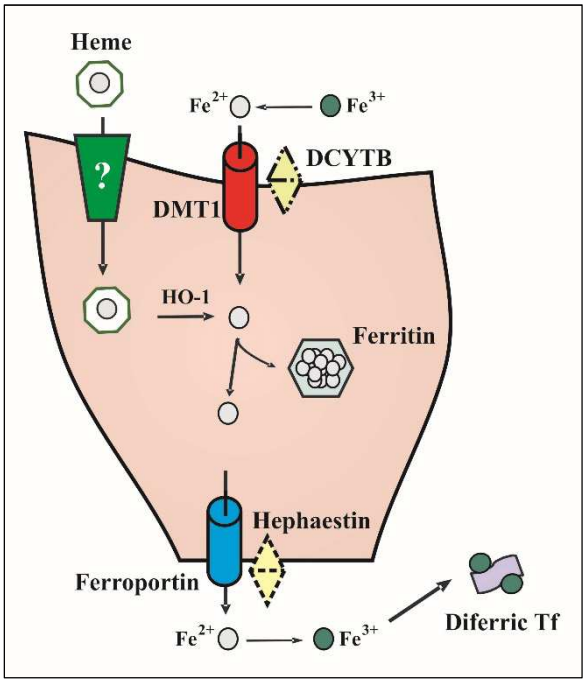

Figure 7. Duodenal iron absorption. Inorganic iron is reduced from Fe^{3+} to Fe^{2+} by duodenal ferrireductases (example DCYTB) and taken up into the cells via DMT1. Iron is then either stored in ferritin or exported to the blood stream via ferroportin. Hephaestin oxidizes Fe^{2+} to Fe^{3+} facilitating its loading onto circulating transferrin (Tf); a complex of diferric transferrin is formed. Heme iron absorption is less understood, but likely involves a putative heme transporter (depicted by ?). Once inside the cell, iron is liberated from PPIX by HO-1 and enters a common pathway with inorganic iron.

On the basolateral membrane of enterocytes ferroportin (FPN, also known as IREG1, MTP1, and SLC40A1) (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000), the only known mammalian iron exporter, mediates iron release to the bloodstream (Donovan et al., 2005). This process is facilitated by multi-copper oxidase hephaestin, which converts Fe^{2+} to Fe^{3+} (Vulpe et al. 1999) and promotes its binding to plasma transferrin (Tf). Each Tf molecule can bind two iron ions to form diferric

transferrin (Fe^{3+}_2-Tf) (Fig. 7). Under normal circumstances 20–45% of Tf binding sites are occupied by iron (measured as percent transferrin saturation - TSAT).

Also TSAT is an important diagnostic marker; it is decreased in iron deficiency and increased when excess amounts of iron are present. In pathological conditions with oversaturated transferrin, non-transferrin-bound iron (NTBI) is present in the circulation and readily taken up by the liver, heart, and other tissues. A fraction of NTBI, so called labile plasma iron (LPI) may catalyze the formation of reactive oxygen species (ROS), which are the main cause of iron-related toxicity (Brissot et al., 2012).

1.2.2 Erythroid iron utilization and soluble transferrin receptor

Erythroid precursors have the greatest need for iron to allow production of large quantity of hemoglobin. Erythroid precursors take up iron through transferrin receptor 1 (TfR1 or TfR) (reviewed in Ponka, 1997), which specifically binds diferric Tf (Fig. 8).

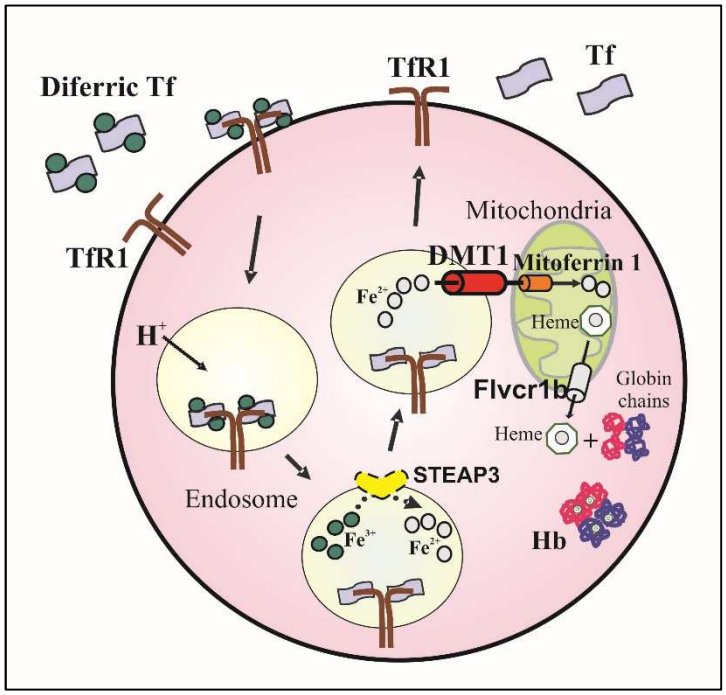

Figure 8. Erythroid iron transport and utilization. Developing erythroblasts take up iron bound to transferrin (diferric Tf) via transferrin receptor 1 (TfR1) mediated endocytosis. Acidification of the endosome (H^+) leads to iron release from the complex. TfR1 and Tf are recycled to the cell surface and blood circulation, respectively. In endosome, ferrireductase STEAP3 reduces Fe^{3+} to Fe^{2+}, which is subsequently exported out of this organelle by DMT1. Mitoferrin (Shaw et al., 2006) presumably delivers Fe^{2+} towards ferrochelatase (FECH) for its insertion into PPIX. Synthesized heme is then transported to cytosol for assembly with globin chains. This step is likely mediated by FLVCR1b.

Only direct delivery of iron to mitochondria as proposed by the „*Kiss and run hypothesisis*" (Sheftel et al., 2007) is depicted. Alternative pathway involves intermediate transport of iron through cytoplasm by chaperons (Shi et al., 2008).

The concentration of the soluble form of TfR (sTfR) in the serum is an indicator of iron status. The levels of sTfR are increased in iron deficiency and decreased in iron-replete states. However, caution needs to be taken in pathological states associated with accelerated erythropoiesis, where elevated sTfR levels reflect stimulated erythropoiesis rather than iron deficiency.

Upon binding, the complex of TfR1/diferric Tf is internalized into endosomal recycling vesicles. Following acidification of endosomes, iron is reduced to ferrous state likely by STEAP3 (six-transmembrane epithelial antigen of the prostate 3) (Ohgami a kol., 2005) to be transported across the endosomal membrane by DMT1 (Fleming et al., 1997) (Fig. 8); the expression of this erythroid specific isoform of DMT1 (isoform II; - IRE/1B) is not regulated by iron.

The process of iron delivery to mitochondria is not completely understood. Nevertheless, once inside the organelle, iron is inserted into PPIX by ferrochelatase to form heme (Ajioka et al., 2006). Finally, to enable the formation of hemoglobin molecule, heme needs to be transported into the cytosol to associate with the globin chains (Fig. 8). In 2004, Quigley et al. reported that feline leukemia virus, type C, receptor 1 (FLVCR1) may act as a heme exporter. Later experiments revealed that the FLVCR1b isoform, expressed in the mitochondria, is essential for heme export out of this organelle (Chiabrando et al., 2012) (Fig. 8). The proposed role of FLVCR1a isoform in heme export out of developing erythroid precursors to avoid cytosolic heme toxicity is still a matter of debate.

Besides heme, iron forms in the mitochondria also iron-sulfur (Fe-S) clusters, which are essential for respiratory chain complexes and a number of mitochondrial and cytosolic proteins (reviewed in Lill and Mühlenhoff, 2008).

1.2.3 Erythrophagocytosis and iron recycling

Erythrophagocytosis is a process essential for iron recycling. Most of the circulating plasma iron (15-25 mg/day) comes from erythroid cells phagocytized by macrophages in the liver, spleen, and bone marrow.

Macrophage ingestion of senescent or damaged erythrocytes leads to their breakdown, degradation of hemoglobin, and iron release from heme by HO-1 (Poss and

Tonegawa, 1997). Iron is then exported from phagolysosomes to the cytosol presumably by DMT1 and its homolog natural resistance associated macrophage protein 1 (NRAMP1) (Jabado et al., 2002; Soe-Lin et al., 2009) and can be either stored in the cytosol (bound to ferritin) or transported back to the circulation by ferroportin (Donovan et al., 2005). Iron binding to Tf is facilitated by a multicopper ferroxidase ceruloplasmin (CP), which oxidizes ferrous iron to ferric iron (Harris et al., 1999) (Fig. 9).

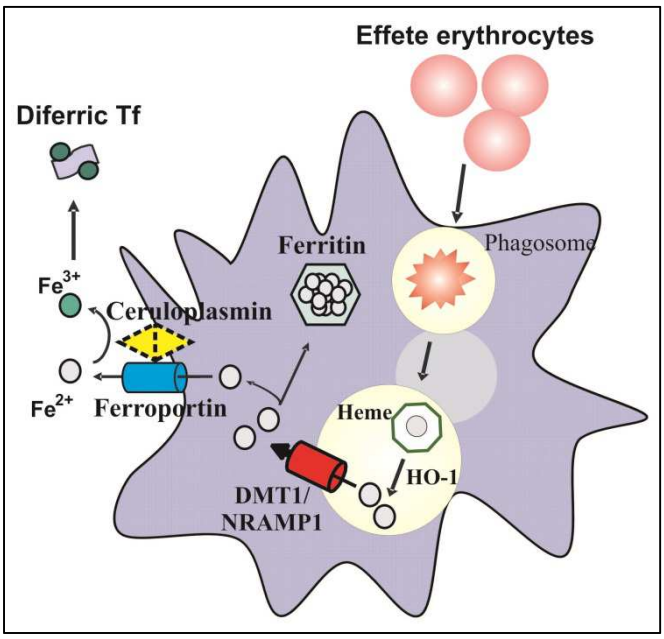

Figure 9. Erythrophagocytosis. Senescent or damaged red blood cells are phagocytosed by macrophages and degraded. Iron is released from heme by HO-1 and exported to cytosol by NRAMP1 or DMT1. Iron is then either stored in ferritin for utilization in times of need or returned to the circulation. Iron is exported from macrophages by ferroportin and oxidized iron (by ferroxidase ceruloplasim) is loaded onto Tf (diferric Tf).

Erythrophygocytosis can be substantially increased due to **ineffective erythropoiesis** (Beguin et al. 1988), which is characterized by an **increased number of erythroid precursors in the bone marrow and concomitant cell death of erythroid precursors and mature erythrocytes**.

Abnormalities in macrophage iron recycling contribute to the development of several pathological conditions particularly anemia of chronic diseases and hereditary hemochromatosis. The underling defect is disruption of iron export from macrophages. An impaired recycling of erythrocyte-derived iron is also responsible for iron overload in heavily transfused patients, such as those with β-thalassemia major (reviewed in Ganz and Nemeth, 2012a).

1.2.4 Regulation of iron homeostasis

The role of iron homeostasis is to keep iron concentration tightly regulated to fulfill the demand for iron while avoiding its toxicity. This happens at organismal (systemic) as well as cellular level; the first involves hepcidin/ferroportin regulatory circuitry and the later action of iron regulatory proteins (IRPs) via their binding to iron response elements (IREs). In addition, hypoxia seems to be playing a hepcidin-independent homeostatic role, as HIFs regulate expression of a number of genes involved in iron metabolism. This is best documented in duodenal enterocytes, where HIF2 controls iron uptake and export by regulating the expression of DCYTB ferric reductase, intestinal DMT1- isoform I (+IRE/1A), ferroportin, and HO-1 (reviewed in Haase, 2013).

1.2.4.1 Cellular iron homeostasis

Cellular iron homeostasis is responsible to ensure adequate, but not excessive iron supply to each cell. The best studied mechanism relies on the action of two cytoplasmic iron regulatory proteins: IRP1 and IRP2. These proteins bind to IREs present in either 5' or 3' untranslated regions (UTRs) of mRNA (Eisenstein and Blemings, 1998) of genes encoding proteins crucial for iron uptake, storage, and heme biosynthesis and thus affect their translation and/or mRNA stability (Cairo and Pietrangelo, 2000). The IRE binding capacity of IRP1 and IRP2 depends on the levels of intracellular iron.

When iron is low, IRP1 and IRP2 bind to IREs leading to stabilization and increased expression of mRNA encoding proteins involved in iron uptake such as TfR1 and intestinal DMT1- isoform I (+IRE/1A), with IREs in their 3'UTR. Simultaneously, the expression of iron storage or export proteins (ferritin L- and H-chains and ferroportin) and the erythroid specific heme biosynthetic enzyme (5'-aminolevulinate synthase 2, ALAS2) is blocked as their mRNA contains IREs in the 5' UTR. The opposite happens in high iron condition; *i.e.* block of iron absorption and enhanced iron storage as IRP1 and IRP2 lose their capacity to bind IREs and the expression of corresponding mRNAs is therefore regulated inversely. More details on IRE/IRP system can be found in the Attachment 7 or in the literature (Muckenthaler et al., 2008).

1.2.4.2 Heme regulatory role in erythroid iron homeostasis

The highest levels of heme are produced in developing erythroid cells. Thank to collaboration with prof. P. Ponka (Montreal, Canada) I contributed on a study analyzing the role of heme-degrading enzyme HO-1 (Poss a Tonegawa, 1997) in erythropoiesis, which was that time not known.

Using cellular models we have demonstrated that HO-1 is expressed in erythroid cells and upregulated during erythroid differentiation. Overexpression of HO-1 causes depletion of heme and leads to a secondary decrease in TfR1 expression and iron uptake by the cell. Moreover, reduction in heme levels results in increased HRI kinase activity which, in turn, represses globin translation via phosphorylation of eIF2α. On the other hand, HO-1 deficiency leads to increased levels of heme resulting in a mainly transcriptional activation of TfR1 expression and an enhanced iron uptake by the cell. The increase in the heme levels also represses HRI activity, leading to augmented globin translation. Thus under physiological conditions, appropriate levels of HO-1 guarantee optimal hemoglobinization rates. We proposed that HO-1 controls the levels of "free" or uncommitted heme pool, serving both precursor and regulatory function (Garcia-Santos et al., 2014).

In 2015 a group of E. Tolosano showed that also FLVCR1 contributes to the control of the size of cytosolic heme in erythroid cells (Mercurio et al., 2015). By modulating and studying FLVCR1 deficiency in mice and zebrafish they proposed that both Flvcr1a and Flvcr1b isoforms are important to ensure proper heme levels for metabolic activity of highly proliferating erythroid progenitors. At this stage export of heme out of the cell by FLVCR1a may be critical to avoid excessive heme accumulation. On the other hand only Flvcr1b, exporting heme from mitochondria to the cytosol, is also required for terminally differentiating erythroid cells to sustain high rate of hemoglobin synthesis. FLVCR1a becomes dispensable and it is plausible, that HO-1 control of heme levels may become important at this time (Garcia-Santos et al., 2014).

1.2.4.3 Systemic iron homeostasis

The mechanisms involved in systemic iron homeostasis regulate iron absorption, deposition, and mobilization from stores and under physiological conditions depend on the erythropoietic activity and amount of stored iron.

The principal regulator of iron homeostasis is a circulating peptide hepcidin produced by liver hepatocytes and cleared by the kidneys. **Hepcidin controls plasma iron concentration by inhibiting dietary iron absorption and release of recycled iron from macrophages and stored iron from hepatocytes** (reviewed in Ganz and Nemeth, 2012b) (Fig. 10).

Hepcidin overexpression causes iron deficiency anemia due to inhibition of iron absorption and iron release from stores (Nicolas et al., 2002a). On the other hand, hepcidin deficiency results in iron hyperabsorption, parenchymal iron overload, and reduction in iron stores within macrophages (Nicolas et al., 2001; Roetto et al., 2003).

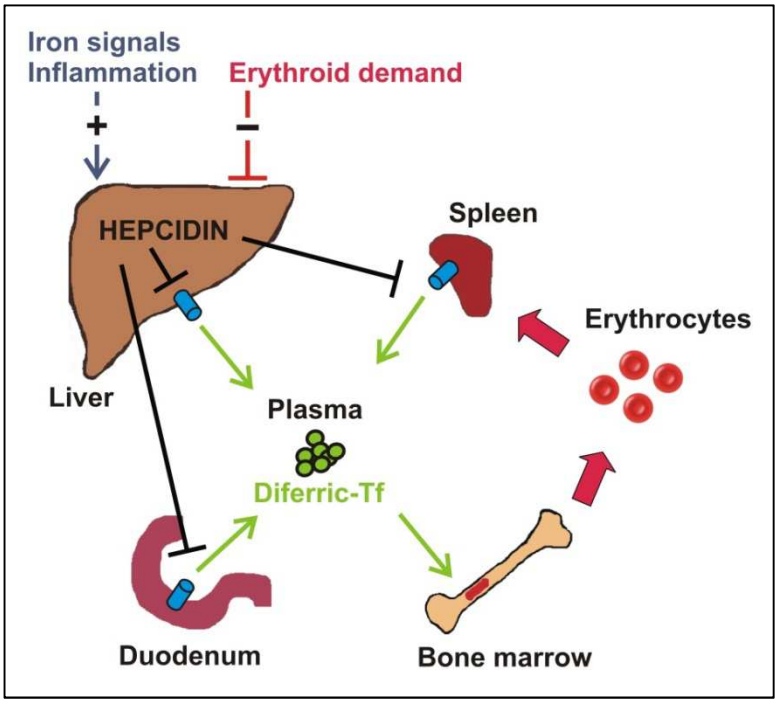

Figure 10. Hepcidin in iron homeostasis. Hepcidin synthesis in hepatocytes is increased in response to high iron and inflammation and suppressed by accelerated erythropoiesis. Hepcidin negatively influences (black lines) export of dietary iron from duodenal enterocytes, release of erythrocyte-derived iron from splenic macrophages, and mobilization of stored iron from hepatocytes by binding to ferroportin (blue cylinder) and inducing its degradation. This consequently leads to the regulation of the iron levels in the circulation (diferric Tf) and its availability for erythropoiesis in the bone marrow.

The action of hepcidin is mediated by its binding to ferroportin, leading to ferroportin internalization and degradation (Nemeth et al., 2004a). Because ferroportin is the sole iron exporter from the cells, its degradation results in the block of iron delivery to the plasma and extracellular fluids and consequently to a drop in iron concentration (Fig. 10).

Hepcidin synthesis is regulated at transcriptional level. It is induced in response to increased levels of diferric Tf in the plasma and increased iron stores in hepatocytes (iron signals) (Corradini et al., 2011) and suppressed during iron deficiency and accelerated erythropoiesis due to anemia, ineffective erythropoiesis (erythroid demand), and/or hypoxia (Nicolas et al., 2002b) (Fig. 10). Hepcidin production is potentially stimulated by inflammation (Nemeth et al., 2003). Recent studies on mice suggested that also growth hormones and factors (Troutt et al., 2012; Goodnough et al., 2012) as well as sex hormones, mainly testosterone (Latour et al., 2014; Guo et al., 2013) and estrogen (Ikeda et al., 2012; Hou et al., 2012; Lehtihet et al., 2016;) may regulate hepcidin levels.

Regulation of hepcidin by iron

Analyses of animal models and studies in patients with genetic diseases (mainly hereditary hemochromatosis - HH and iron refractory iron deficiency anemia – IRIDA) have helped to identify proteins involved in iron-related regulation of hepcidin expression (Table 1, Fig. 11). Please see Attachments 7, 8 and 9 or review by Ganz and Nemeth (2012a,b) for detailed description of these diseases.

Table 1. Genetic abnormalities in proteins involved in the regulation of iron homeostasis identified in humans.

GENE	PROTEIN	FUNCTION	DISEASE	HEPCIDIN LEVELS	FINDINGS/ SYMPTOMS
HFE	**HFE**	Modulator of circulating iron sensing	HH type 1	↓	Fe deposition in the liver, heart, and endocrine tissues
HJV	**HJV**	BMPs co-receptor, activation of BMP/SMAD pathway	HH type 2a, juvenile	↓↓↓	Early onset, severe Fe deposition in the liver, heart, and endocrine tissues
HAMP	**Hepcidin**	Regulator of systemic iron homeostasis	HH type 2b, juvenile	absent	
TfR2	**TfR2**	Sensor of circulating iron	HH type 3	↓↓	Fe deposition in the liver, heart, and endocrine tissues
TMPRSS6	**Maptriptase 2**	Cleavage of mHJV, supression of BMP/SMAD pathway	IRIDA	↑↑	Hypochromic microcytic anemia
SLC40A1	**Ferroportin**	Iron exporter, hepcidin target	HH type 4a	↑↑	Predominant macrophage Fe overload, occasionally anemia
			HH type 4b	↑↑	Parenchymal Fe overload

Table legend: HH - hereditary hemochromatosis, IRIDA - iron refractory iron deficiency anemia, ↓ - decreased, ↑ - increased

In a current model, circulating iron (diferric Tf) is sensed by transferrin receptor 2 (TfR2) and TfR1 assisted by a protein associated with hemochromatosis type I (HFE). TfR2 is stabilized by diferric Tf and increased levels of diferric Tf concomitantly promote displacement of HFE from TfR1 and its binding to TfR2. It is proposed that HFE/TfR2 complex somehow potentiates bone morphogenetic protein (BMP) receptor signaling (reviewed in Ganz and Nemeth, 2012a) (Fig. 11).

In mice, BMP6 acts as an endogenous hepcidin regulator that activates BMP/SMAD signaling (Andriopoulos et al., 2009). BMP6 expression is stimulated by increased hepatic iron stores rather than by circulating iron. BMP6 binds to the type I and type II BMP receptors (BMPR I-II). The membrane-associated form of hemojuvelin (mHJV) acts as a co-receptor for BMPs and via activation of SMAD1-5-8 and SMAD4

transcription factors directly stimulates hepcidin gene expression (Wang et al., 2005) (Fig. 11).

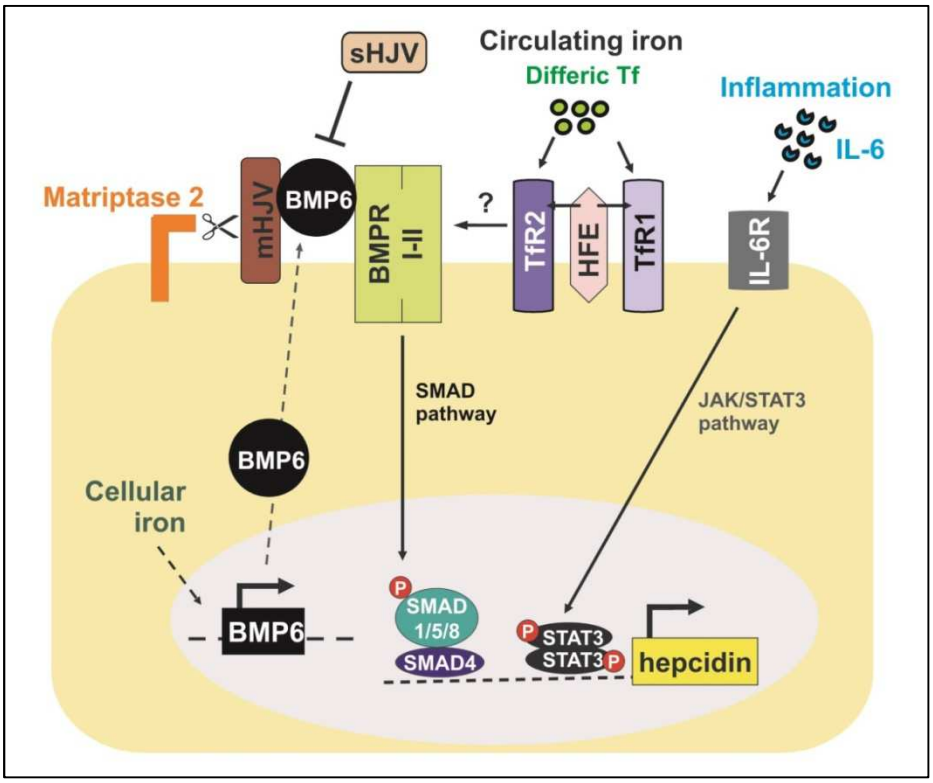

Figure 11. Regulation of hepcidin synthesis. BMP6 expression is stimulated by increased hepatic iron stores (dash line). BMP6 forms a complex with the type I and type II BMP receptors (BMPR I-II) and the co-receptor membrane-bound hemojuvelin (mHJV). This leads to phosphorylation of SMAD 1/5/8, which subsequently binds to SMAD4. The heteromeric SMAD complex translocates to the nucleus, binds to the hepcidin gene promoter, and activates its expression. Soluble hemojuvelin (sHJV) competes with mHJV for BMP6 and thus inhibits hepcidin activation mediated by the BMP6/SMAD pathway. Hepcidin expression is also negatively regulated by the protease matriptase-2; it is presumed that matriptase-2 cleaves mHJV. Hepcidin expression is stimulated also by circulating iron; diferric transferrin stabilizes TfR2, which in complex with HFE likely activates BMPR/SMAD pathway. The precise signaling responsible for this activation remains to be identified (question mark). During inflammation, IL-6 induces hepcidin expression directly via JAK/STAT3 pathway.

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On the other hand, membrane associated serine protease matriptase-2 encoded by *TMPRSS6* (type II transmembrane serine protease 6) (Du et al., 2008) acts as a negative hepcidin regulator. Matriptase 2 cleaves mHJV and thus abrogates the BMP6/SMAD signaling (Silvestri et al., 2008a) (Fig. 11). It was shown that matriptase 2 is stimulated by acute iron deficiency and hypoxia and repressed by iron-rich diet and BMP6. In addition the BMP6/SMAD pathway is also inhibited by soluble HJV (sHJV), which likely competes with mHJV for BMP6 binding (Lin et al., 2005) and by neogenin (Enns et al., 2012) and furin (Silvestri et al., 2008b), but the exact mechanisms are less clear.

Regulation of hepcidin by erythropoiesis

In comparison to iron-related regulation of hepcidin its regulation by erythropoiesis is less clarified. It has been presumed that the erythroid regulators of iron absorption and recycling exist (these were later modified to regulators of hepcidin) in order to adjust iron supply to the production of erythrocytes (Finch, 1994). This becomes even more important in the states of accelerated erythropoietic activity (due to bleeding, hemolysis, ineffective erythropoiesis, and/or hypoxia).

Initially, hypoxia and EPO were proposed to induce hepcidin directly. The direct effect of EPO was excluded using mouse models with disrupted erythropoiesis that were unable to attenuate hepcidin synthesis in response to EPO administration (Pak et al, 2006; Vokurka et al, 2006). Our own work on patients with pyruvate kinase deficiency and mainly Diamond-Blackfan anemia, confirmed these results (see Chapters 2.2.2 and 2.2.3 for details). Similarly, the analyses on mice with modulated HIF activity showed that hepcidin supression by hypoxia requires EPO-induced erythropoiesis (Liu et al., 2012; Mastrogiannaki et al., 2012).

There is therefore now believed that the negative regulation of hepcidin relies on the action of one or more erythroid suppressors, which are produced by EPO-stimulated bone marrow erythroid cells.

Two candidate molecules were proposed as hepcidin suppressors in disease states associated with ineffective erythropoiesis: growth differentiation factor 15 (GDF15) based on a negative correlation with hepcidin levels in human patients with β-thalassemia (Tanno et al., 2007) and twisted gastrulation (TWSG1) based on elevated TWSG1 mRNA in

murine β-thalassemia models (Tanno et al., 2009). However, subsequent research has questioned their role. No induction of TWSG1 in mice injected with EPO or challenged by blood loss indicates that TWSG1 is not involved in physiological suppression of hepcidin (Liu et al., 2012; Casanovas et al., 2013). Similarly, comparable levels of hepcidin in wild-type mice and Gdf15 knock-out mice after phlebotomy and the presence of normal GDF15 levels in blood donors indicate that neither GDF15 is involved in the physiological suppression of hepcidin (Casanovas et al., 2013). In addition, the levels of TWSG1 in human patients with ineffective erythropoiesis are unknown. Finally, recent study suggested that GDF15 should rather be considered as a marker of ineffective erythropoiesis (Ronzoni et al., 2015). Our own data presented in Chapters 2.2.2, 2.2.3, and 2.2.4 are in agreement with this conclusion.

Very promising seems to be a recent description of an erythroid regulator, named erythroferrone (ERFE) (Kautz et al., 2014). ERFE is encoded by *Fam132b* gene and was previously described as myonectin or CTRP15, a member of the C1q/tumors necrosis factor (TNF)-related protein family (CTRP). ERFE is produced by erythroid precursors in response to EPO administration via JAK2/STAT5 pathway. Although the adult ERFE knock-out mice have normal hematological and iron status parameters they exhibit a delay in recovery from blood loss due to a failure of acute hepcidin suppression. In addition, β-thalassemia intermedia mouse model shows elevated ERFE expression (Kautz et al., 2015), suggesting its role in hepcidin suppression in ineffective erythropoiesis. However, further research is needed to clarify ERFE role in the regulation of hepcidin production.

Although the identity of pathological and especially physiological erythroid regulators of hepcidin remains elusive it is well accepted that these erythroid signals have stronger effect on hepcidin synthesis than iron-related signals. This is best documented on pathological conditions such as β-thalassemia and congenital dyserythropoietic anemia, where ineffective erythropoiesis is accompanied by hyperferremia, iron overload in parenchymal tissues, and very low or nearly undetectable levels of hepcidin (Papanikolaou et al., 2005). Suppressed hepcidin despite massive iron overload clearly shows that signals controlling iron supply for erythroid cells dominate over iron-sensing signaling.

Regulation of hepcidin by inflammation

Pathological conditions associated with infection and inflammation are characterized by substantial increase in hepcidin levels, which supports the antimicrobial role of hepcidin. The key molecule stimulating hepcidin synthesis is interleukin-6 (IL-6) (Nemeth, et al., 2004b) which activates hepcidin expression via JAK/STAT3 pathway (Wrighting and Andrews, 2006) (Fig. 11). Recent studies indicated that during inflammation JAK/STAT3 pathway induces hepcidin synthesis synergistically with BMP/SMAD signaling (activated by activin B or BMP family members) (Verga Falzacappa et al., 2008; Canali et al. 2016). IL-6 is produced by neutrophils, Kupffer cells, and many other cells and this direct activation of hepcidin results in decreased availability of iron and is the pathophysiological mechanism responsible for anemia of chronic diseases (Nemeth and Ganz, 2014).

1.3 Inherited defects of erythropoiesis

Disorders of erythropoiesis can be classified as anemia or polycythemia and the corresponding abnormalities may be either inherited or acquired.

1.3.1 Anemia

Anemia is a state characterized by decreased level of hemoglobin, which can be accompanied by reduced number of erythrocytes in the circulation. This consequently leads to inappropriate oxygen supply and tissue hypoxia. The most common cause is acquired nutritional iron deficiency known as iron deficiency anemia (IDA) resulting in hypochromic and microcytic erythrocytes. IDA needs to be distinguished from inherited microcytic anemias, as it can be successfully treated with iron supplements. On the other hand, iron therapy is usually inappropriate for inherited microcytic anemias caused by a defect in iron metabolism or hemoglobin production (see Attachment 8 for details).

Inherited anemias can be classified, according to the causative pathophysiology, into anemias caused by **1) impaired production of erythrocytes in the bone marrow** or **2) increased destruction of erythrocytes.**

1.3.1.1 Inherited anemias due to impaired production of erythrocytes

Impaired production of erythrocytes may have different reasons:

A) Defect in proliferation and differentiation that can affect either erythroid cells or hematopoietic stem cells. The first group includes **Diamond-Blackfan anemia (DBA)**, a pure red cell aplasia and **congenital dyserythropoietic anemia (CDA)** characterized by premature destruction of erythroblasts. The second group is represented by **Fanconi anemia**, which is an aplastic anemia with concomitant occurrence of leukopenia and thrombocytopenia caused by bone marrow failure.

B) Defect in globin synthesis (*hemoglobinopathies*) that may be related to the production of a structurally abnormal hemoglobins; i.e **sickle cell anemia** or to an imbalance in globin chain synthesis leading to **thalassemia** (mainly β- or α-thalassemia).

C) Disrupted heme synthesis which is typical for **sideroblastic anemia** and is associated with the accumulation of iron in mitochondria of developing erythroblasts. This category also includes **rare inherited defects of iron transport** resulting in iron-deficient erythropoiesis; an example is the anemia due to DMT1 deficiency (see Chapter 2.1 and Attachment 7 and 8 for details).

D) Disrupted synthesis of DNA that is characteristic for **megaloblastic anemias** including pernicious anemia. Typical are, besides the abnormalities in erythropoiesis, also defects in granulopoiesis and thrombopoiesis leading to pancytopenia. Although the vast majority of cases are acquired, a small number may be caused by inherited defects.

1.3.1.2 Inherited anemias due to increased destruction of erythrocytes - hemolytic anemia

In this second group of anemias, the underlying cause is increased destruction of erythrocytes (hemolysis). Hemolytic anemias result from increased breakdown of erythrocytes, which is not completely compensated by the enhanced erythropoiesis in the bone marrow. Enhanced erythropoiesis leads to an increased release of reticulocytes into the circulation, making the high reticulocyte count one of the diagnostic criteria for hemolysis.

In the case of inherited hemolytic anemia, the causative defect is intrinsic to erythrocytes and may be due to:

A) Defect of erythrocyte membrane production leading to formation of abnormally shaped erythrocytes, most commonly spherocytes in **hereditary spherocytosis** (HS) and elliptocytes in **hereditary elliptocytosis** (HE).

B) Defect in erythrocyte metabolism due to an abnormal activity of erythrocyte enzymes such as pyruvate kinase (PK) or glucose-6-phosphate dehydrogenase (G6PD) leading to **PK deficiency** and **G6PD deficiency**, respectively.

C) Defect in hemoglobin production leading to **hemoglobinopathies with unstable hemoglobins**. In some of the already mentioned hemoglobinopathies, such as sickle cell anemia, a hemolytic crisis may develop.

1.3.1.3 α- and β-thalassemia

Our own work presented in Chapter 2.2 has contributed to the characterization of an abnormal crosstalk between erythropoiesis and iron metabolism in the different types of inherited anemias.

Because β-thalassemia intermedia and major represent model disorders to study disrupted balance between erythropoiesis and iron metabolism, as it will be discussed repeatedly in the Chapter 2.2, more details will be given with respect to the pathophysiology of these diseases.

Thalassemias are a heterogeneous group of inherited disorders characterized by reduced or absent synthesis of one or several globin chains which are a part of the hemoglobin molecule. Under physiological conditions, the ratio of α- and non-α-globin chains (predominantly β-globins in adults) in erythrocytes is balanced (1:1). The pathophysiology and clinical symptoms of thalassemia are related to the extent of the imbalance between α- and β-globin chain synthesis and reflect the severity at the molecular level; mutant alleles causing more profound imbalance in the globin production would be associated with more severe alteration in erythropoiesis. The causative mutations may either lead to reduced (β^+, α^+) or absent synthesis (β^0, α^0) of globin chains (reviewed in Weatherall, 2001).

The majority of β-thalassemia mutations are single nucleotide substitutions, deletions, and insertions of oligonucleotides affecting the coding sequence as well as intronic and promotor region of β-globin gene (Murru et al., 1991; Huisman, 1997; Forget, 2001). More rarely, β-thalassemia results from β-globin gene deletions or deletion of the regulatory LCR (Popovich et al., 1986; Forget, 2001).

Heterozygous carriers of a β-thalassemia allele (β^+/β or β^0/β) are usually asymptomatic, with hypochromic and microcytic erythrocytes, mild anemia accompanied by an compensatory increase in the number of red blood cells (erythrocytosis) and slightly increased erythropoiesis in the bone marrow. This type of thalassemia is known as **β-thalassemia minor** or **β-thalassemia trait** (Weatherall, 2001). The information on disrupted erythropoiesis and iron metabolism in β-thalassemia trait is just being published (see Chapter 2.2.4 and Attachment 6)

More profound imbalance in α- and β-globin chain synthesis (β^+/β^+ or β^+/β^0) leads to **β-thalassemia intermedia**. Patients exhibit moderate anemia with signs of hemolysis, ineffective erythropoiesis, and splenomegaly. Some patients may require occasional transfusions (Weatherall, 2001). It is known that hepcidin levels are low in β-thalassemia intermedia due to the suppressive effect of accelerated erythropoiesis, which leads to the hyperabsorption of dietary iron and development of secondary iron overload independently of transfusions (Nemeth, 2010). Several molecules has been shown to correlate negatively with hepcidin in β-thalassemia patients or mouse model, i.e. GDF15 (Tanno et al., 2007), TWSG1 (Tanno et al., 2009), and ERFE (Kautz et al., 2015), but their role in hepcidin attenuation (as it was discussed above) needs to be clarified. Increased iron levels in the circulation cause oversaturation of transferrin, and thus concomitant increase of NTBI in the plasma. NTBI is rapidly taken up by the hepatocytes and other parenchymal cells (cardiac myocytes and endocrine cells) causing tissue injury and organ damage (Origa et al., 2007; Nemeth, 2010).

The most severe form of β-thalassemia is **β-thalassemia major** (β^0/β^0). Hemoglobin levels at diagnosis may be below 3.0 g/dL (normal range: 12.0 – 15.0 g/dL) and patients require regular transfusions (Weatherall, 2001). Hepcidin levels are higher than in β-thalassemia intermedia, likely due to the suppressive effect of transfusions on erythropoietic drive (Origa et al., 2007; Nemeth, 2010). Higher hepcidin is responsible for preferential iron deposition in the macrophages. Nevertheless, it was shown that in the intervals between transfusions, hepcidin levels gradually decrease, allowing increased iron absorption in the duodenum, which thus contributes to iron overload (Pasricha et al., 2013).

The clinical picture of α-thalassemia is dependent on the degree of reduction of α-globin chain synthesis; i.e. on the total number of mutated α-globin genes. Four α-globin genes are present in normal diploid human genome (αα/αα) and these may be affected by deletions (most commonly) or rarely by inactivating point mutations. Individuals with deletion/inactivation of one allele (-α/αα) are asymptomatic and considered **silent carriers** (reviewed in Piel and Weatherall, 2014).

α-thalassemia minor or **α-thalassemia trait** is typical for individuals who inherit two mutant α-globin alleles (--/αα or -α/-α). These individuals are usually clinically asymptomatic with mild hypochromic microcytic anemia and slightly increased red blood cell count (Piel and Weatherall, 2014).

Hemoglobin H disease results from mutations affecting three α-globin genes (--/-α) and presents with moderate to severe anemia and splenomegaly. The inactivation of all four α-globin genes (--/--) is incompatible with life and leads to fetal death - **hydrops fetalis** (Piel and Weatherall, 2014).

The data on disrupted hepcidin-erythropoiesis axis in α-thalassemia are limited. A pilot study on α-thalassemia carriers has only recently been published (Guimarães et al., 2015).

1.3.2 Polycythemia

The true polycythemia (or erythrocytosis) is characterized by increased red cell mass, which can be accompanied by elevated hemoglobin, hematocrit, and increased number of red blood cells (Berlin, 1975).

In **primary polycythemias**, inherited germline mutations or acquired somatic mutations that are expressed within the hematopoietic progenitors lead to their augmented responsiveness to EPO (i.e. EPO-hypersensitivity or EPO independence). The patients exhibit reduced/low-normal EPO levels (reviewed in Prchal, 2010).

The only known **germline mutations** in primary polycythemia are **EPOR** truncations leading to EPOR gain-of-function. These cases are referred to as **primary**

familial and congenital polycythemia (**PFCP**) (Kralovics and Prchal, 2000). The best characterized **somatic mutation causing acquired primary polycythemia is the V617F substitution in JAK2**, a hallmark of polycythemia vera (**PV**) (Fig. 12). (Baxter et al., 2005; James set al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005) Additional somatic JAK2 mutations in V617F-negative patients were reported in JAK2 exon 12 (Scott et al., 2007).

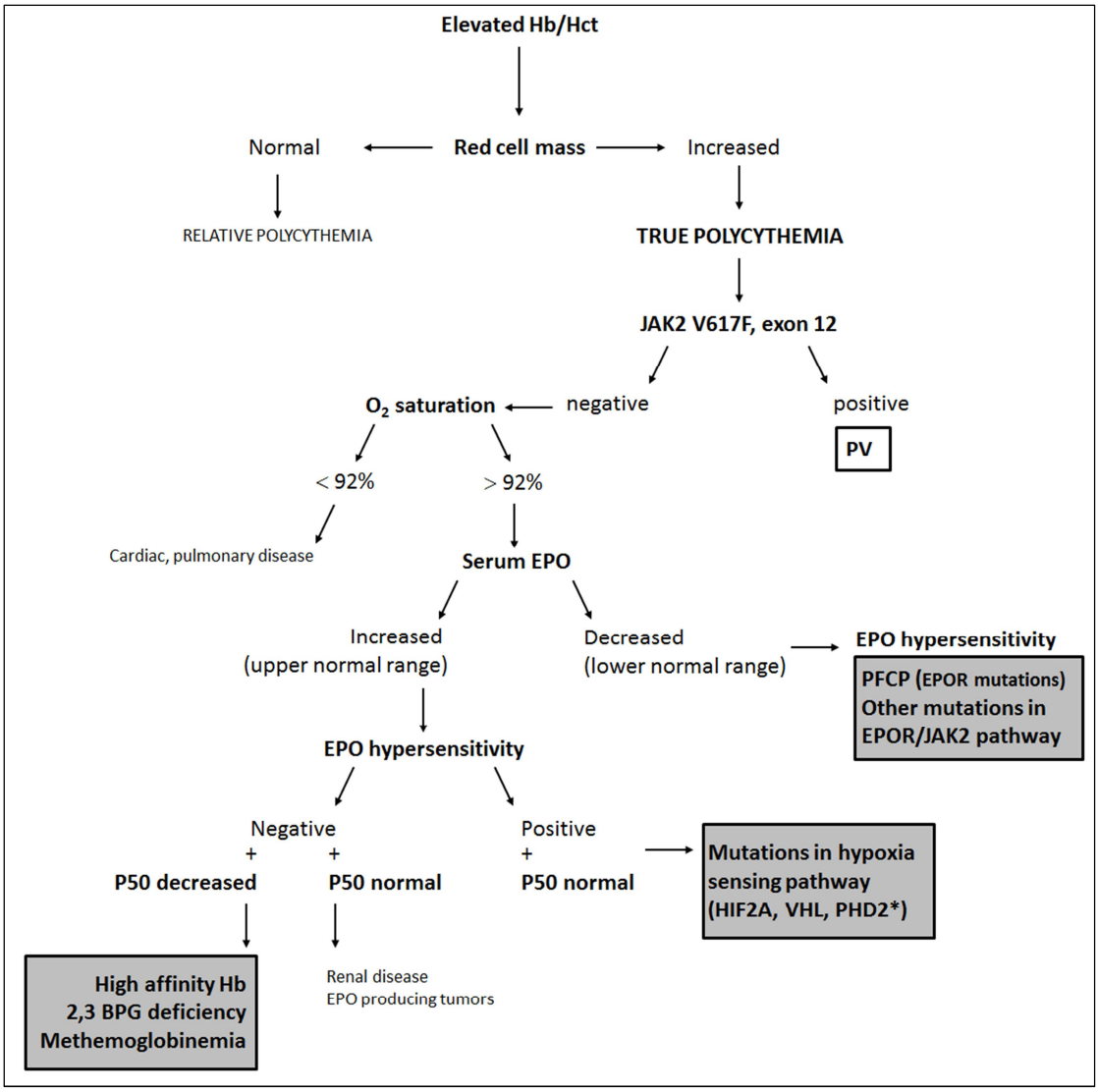


Figure 12. Differential diagnosis of polycythemia states. The inherited causes are depicted in grey rectangles. * - the information on EPO hypersensitivy in PHD2-mutant cases is incomplete. For details please see the main text. Adapted and modified from Spivak and Silver, 2008 and Patnaik and Tefferi, 2009.

The consequences of genetic lesions in EPOR and JAK2 include mainly the alterations of the JAK/STAT signaling pathway, with STAT5 being the most critical downstream effector of EPOR/JAK2 signaling (Fig. 3) (Constantinescu et al., 1999). In contrast to patients with neoplastic PV, where oncogenic JAK2 V617F drives constitutive activation of JAK2/STAT5 (Funakoshi-Tago et al., 2010), the patients with non-malignant PFCP reveal only prolonged activation of JAK2/STAT5 pathway (Arcasoy et al., 1999; Divoky et al., 2016), do not develop clonal disease, and do not transform to acute leukemia (Kralovics and Prchal, 2000; Prchal 2010).

Recently, mutations in **LNK**, the negative regulator of EPOR/JAK2 signaling, have been reported in idiopathic erythrocytoses (cases with unidentified molecular defect) and subnormal or normal EPO levels (reviewed in McMullin and Cario, 2016). The functional consequences of these mutations are not completely known; nevertheless some of them have been reported also in patients with myeloproliferative neoplasm (MPN) and shown to cause improved cellular proliferation (reviewed in McMullin and Cario, 2016). MPNs represent a heterogeneous group of diseases, including PV, with an excessive clonal production of one or more types of blood cells (red, white or platelets) in the bone marrow that may evolve into acute leukemia (Tefferi et al, 2009).

Secondary polycythemias are caused by increased levels of erythropoiesis stimulating factors such as EPO, insulin growth factor 1 (IGF-1), or cobalt. These extrinsic factors stimulate erythropoiesis either in response to hypoxia or in excess of the physiological needs. The characteristic features are elevated EPO levels (or inappropriately normal for elevated hematocrit) and normal erythroid response of erythroid progenitors to EPO (reviewed in Prchal, 2010).

Acquired secondary polycythemia may be related to hypoxia caused by cardiac or pulmonary disease or to increased production of EPO in renal disease or by certain tumors. Cardiac or pulmonary disease are suspected when the arterial oxygen saturation is below 92% (< 92%) (Fig. 12).

Polycythemia which develops as an adaptation to high altitude or in tobacco use also belongs to the category of acquired secondary polycythemias which are driven by hypoxia (reviewed in Prchal, 2010).

Inherited secondary polycythemia can be caused by the presence of **high oxygen affinity hemoglobin** due to **mutations in α- or β-globin genes** or by **loss-of-function mutations in 2,3-bisphosphoglycerate (2,3-BPG) mutase.** 2,3-BPG mutase is a glycolytic enzyme which modulates the level of 2,3-BPG. 2,3-BPG deficiency decreases tissue release of oxygen and results in a physiologically appropriate increase in EPO levels (reviewed in Prchal, 2010). The assessment of hemoglobin oxygen dissociation kinetics and calculation of P50 values (the partial pressure of O_2 at which hemoglobin becomes 50% saturated) is a useful test in differential diagnosis of polycythemia states (Fig. 12 and 13) (Patnaik and Tefferi, 2009). The leftward shift of dissociation curve and decreased P50 may indicate the presence of one of the above mentioned abnormalities and further molecular testing of these potential targets is then advised (Fig. 13).

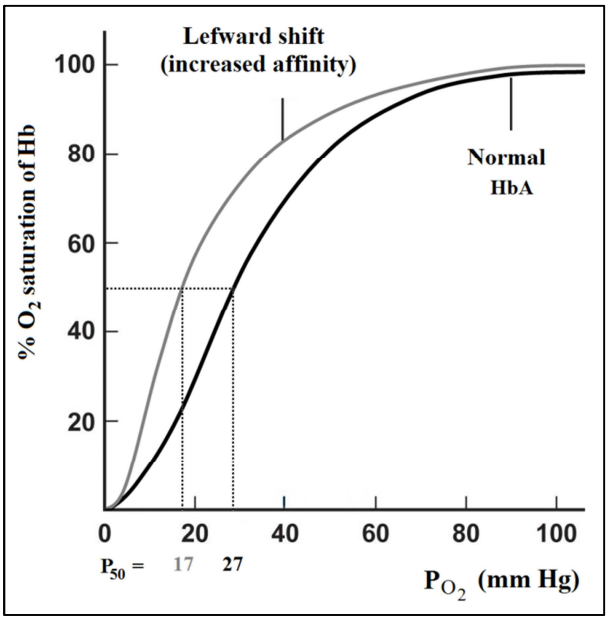


Figure 13. Oxygen dissociation curve. P50 values represent the partial pressure of O_2 at which hemoglobin is saturated at 50%. P50 in a healthy person with normal Hb is 26 ± 1.3 mm Hg.; P50 < 20 mm Hg is considered as abnormaly low. Adapted and modified from Patnaik and Tefferi, 2009.

Rare **inherited methemoglobinemias** (for example due to hemoglobin M or cytochrome b5 reductase deficiency) may lead to a compensatory polycythemia in response to hypoxia which develop due to decreased oxygen carrying capacity and/or decreased oxygen release into the tissues. Increased methemoglobin level (above 1%) is present in these patients (reviewed in Prchal, 2010).

If the P50 values and oxygen dissociation curve are normal **germline mutations in genes involved in the hypoxia-sensing pathway are suspected** (Fig. 12). **These include mutations in VHL, HIF2A,** and **PHD2**, which inappropriately increase signaling mediated by HIF and cause overproduction of EPO (Patnaik and Tefferi, 2009). Some of the VHL- and HIF2A-mutant erythroid progenitors exhibit hypersensitivity to EPO and thus share some features with primary polycythemia states (Kapralova et al., 2014); the information on EPO responsiveness in PHD2-mutant progenitors is incomplete.

Despite advances in this research area a considerable number of patients are still classified as **idiopathic erythrocytosis as the exact cause of polycythemia remains unknown**.

It is believed that patients with low EPO levels (or in lower normal range) have an abnormality in EPOR pathway, while individuals with elevated EPO (or in upper normal range) have abnormality in HIF signaling.

2. COMMENTARY TO THE COLLECTION OF SELECTED PUBLICATIONS

My habilitation thesis is based on previous research of our group, lead by Dr. Vladimir Divoky. The thesis represents a direct continuation of my Ph.D. work, which was focused on cellular and molecular characterization of selected congenital defects of erythropoiesis. The articles presented in my habilitation thesis are divided into three areas:

2.1 Functional consequences of DMT1 deficiency on erythropoiesis

2.2 Disordered iron homeostasis in congenital defects of erythropoiesis

2.3 Molecular pathophysiology of inherited erythrocytoses

The data and experiments presented in Chapters 2.1 and 2.3 were performed with the contribution of my Ph.D. students: Zuzana Zidova, Katarina Kapralova, and Barbora Kralova and in part (Chapter 2.3) in collaboration with the group of prof. Josef T. Prchal from Utah.

The analyses presented in Chapter 2.2 were done in collaboration with the clinicians from hematology centers in the Czech and Slovak Republics, especially prof. Dagmar Pospisilova from the Department of Pediatrics, at the University Hospital Olomouc. The proteomic assay for hepcidin measurements was established at the Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University Olomouc. Renata Mojzikova and Pavla Koralkova from the Department of Biology Faculty of Medicine and Dentistry, at Palacky University Olomouc established the tests for the detection of PK-deficiency and the measurements of erythrocyte enzyme activities. Martina Divoka from the Department of Hemato-Oncology, at the University Hospital Olomouc was responsible for the diagnostic testing of erythrocyte membrane defects and thalassemias.

2.1 Functional consequences of DMT1 deficiency on erythropoiesis

DMT1 is a transmembrane protein, which plays a crucial role in iron metabolism. It mediates a pH-dependent ferrous iron uptake from the gut lumen into enterocytes in the proximal duodenum and iron transport from the transferrin-transferrin receptor complex towards the mitochondria, the site of heme synthesis in erythroblasts (Gunshin et al., 1997; Canonne-Hergaux et al., 1999; Touret et al., 2003).

The G185R missense mutation in DMT1 leads to hypochromic microcytic anemia in two rodent models: so called „*microcytic anemia*" (*mk/mk*) mice (Fleming et al., 1997) and „*Belgrade*" (*b/b*) rats (Fleming et al., 1998). Targeted disruption of the gene (*Slc11a2*) coding for DMT1 causes early postnatal lethality (before postnatal day 7) related to the failure of effective erythroid iron utilization and severe anemia (Gunshin et al., 2005b).

Our group has described the first human mutation in DMT1 (Priwitzerova et al., 2004, Mims et al., 2005, Priwitzerova et al., 2005). Functional analyses showed that both the expression and function of DMT1 protein are impaired by this mutation. **Disruption of erythroid iron transport due to DMT1 deficiency represented a novel mechanism of congenital hypochromic microcytic anemia in humans.** In contrast to *mk/mk* mice (strain MK/ReJ), majority of DMT1-mutant patients presented with increased serum iron and hepatic iron overload. Interestingly, high doses of recombinant human EPO (darbepoietin) ameliorated severe anemia in our DMT1-mutant patient (Pospisilova et al., 2006). Similar positive results of high-dose EPO therapy were also reported for the second Italian DMT1-mutant patient (Iolascon et al., 2008).

Two original manuscripts are presented in this section. The following commentary summarizes the most important results.

1. **Horvathova M**, Kapralova K, Zidova Z, Dolezal D, Pospisilova D, Divoky V. Erythropoietin-driven signaling ameliorates the survival defect of DMT1-mutant erythroid progenitors and erythroblasts. ***Haematologica***. 2012; 97:1480-8. (Attachment 1)

2. Zidova Z, Kapralova K, Koralkova P, Mojzikova R, Dolezal D, Divoky V, **Horvathova M**. DMT1-mutant erythrocytes have shortened life span, accelerated

glycolysis and increased oxidative stress. ***Cell Physiol Biochem***. 2014; 34:2221-31. (Attachment 2)

2.1.1 Erythropoetin therapy in DMT1 deficiency (Attachment 1)

In this study we analyzed the bases for the clinical success of EPO treatment in anemia caused by DMT1 deficiency. For this purpose, we used primary cells of the Czech DMT1-mutant patient and DMT1-mutant mice propagated on a novel 129S6/SvEvTac background (Gunshin et al, 2005b) provided by Dr. Fleming from Boston.

We revealed that the 129S6/SvEvTac-DMT1-mutant mice exhibit iron restricted erythropoiesis and increased plasma iron. This phenotype is reminiscent of the phenotype of DMT1-mutant patients, but differs from the originally described *mk/mk* mice strain MK/ReJ (Fleming et al., 1997) with documented hyposideremia (Bannerman et al., 1972). In this regard, these 129S6/SvEvTac-DMT1-mutant mice seem to be more accurate model for comparison with patients' samples. In agreement with patient´s data, flow cytometry analysis of the erythroid differentiation showed predominance of immature erythroblasts in the bone marrow and spleen of DMT1-mutant mice when compared to wild-type (wt) mice.

In vitro colony assay and immunofluorescence detection of apoptosis (by TUNEL assay) using patient´s samples before and after EPO therapy showed improved survival of erythroid cells following EPO treatment; i.e. improved growth of BFU-E colonies and reduced number of apoptotic erythroblasts (Fig. 1).

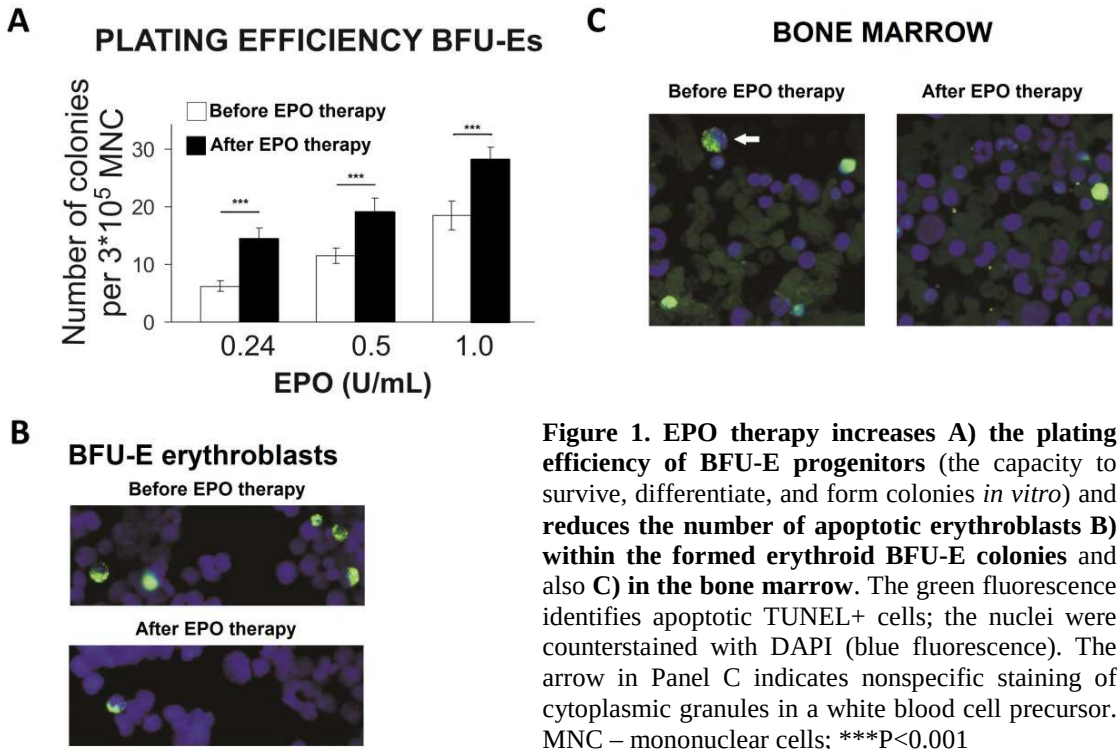


Figure 1. EPO therapy increases A) the plating efficiency of BFU-E progenitors (the capacity to survive, differentiate, and form colonies *in vitro*) and **reduces the number of apoptotic erythroblasts B) within the formed erythroid BFU-E colonies** and also **C) in the bone marrow**. The green fluorescence identifies apoptotic TUNEL+ cells; the nuclei were counterstained with DAPI (blue fluorescence). The arrow in Panel C indicates nonspecific staining of cytoplasmic granules in a white blood cell precursor. MNC – mononuclear cells: ***P<0.001

To assess the effect of EPO treatment in more details, high doses of EPO were administered to DMT1-mutant mice. EPO administration to DMT1-mutant mice led to augmented STAT5 activation, increased expression of two anti-apoptotic proteins (BCL-XL and MCL-1), and consequently to reduced apoptosis of erythroblasts (Fig. 2).

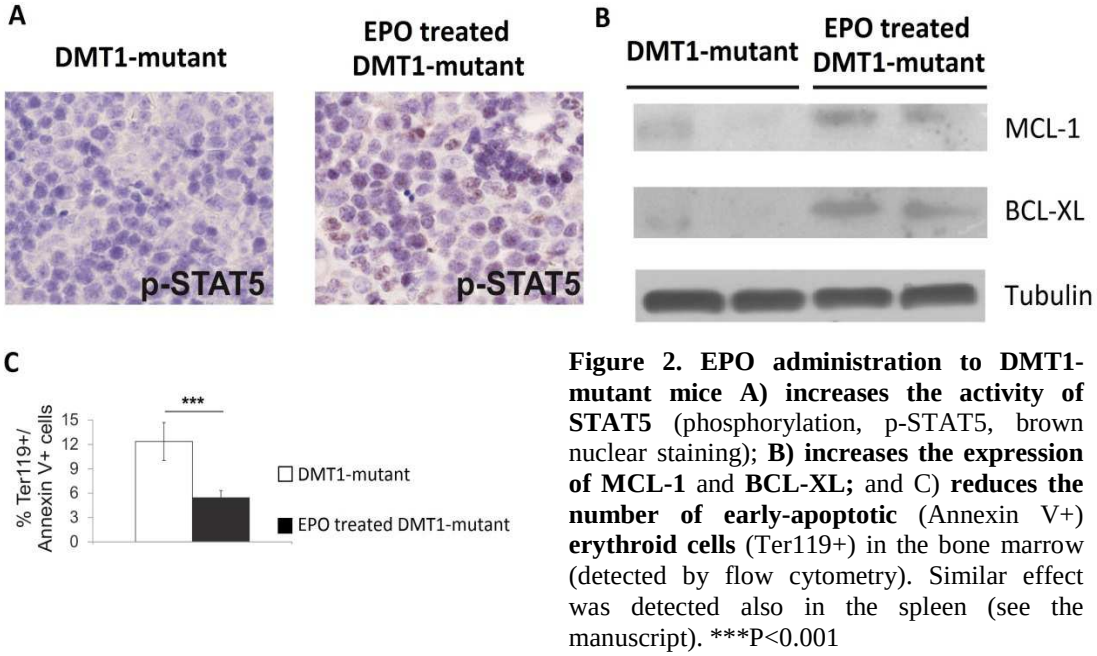


Figure 2. EPO administration to DMT1-mutant mice A) increases the activity of STAT5 (phosphorylation, p-STAT5, brown nuclear staining); **B) increases the expression of MCL-1** and **BCL-XL;** and C) **reduces the number of early-apoptotic** (Annexin V+) **erythroid cells** (Ter119+) in the bone marrow (detected by flow cytometry). Similar effect was detected also in the spleen (see the manuscript). ***P<0.001

Finaly, we also assessed the systemic iron homeostasis in our DMT1-mutant patient and DMT1-mutant mice. Low to undetectable expression of hepcidin confirmed an inadequate hepcidin suppression by increased erythropoietic activity. The role of GDF15 and TWSG1 in hepcidin attenuation in DMT1 deficiency was not confirmed.

Based on our results we propose that the **inhibition of apoptosis of erythroid progenitors and differentiating erythroblasts plays a crucial role in the success of EPO therapy under conditions of DMT1-deficient erythropoiesis**. We can also conclude that the **ineffective erythropoiesis** associated with iron-deficient anemia due to partial loss-of-function DMT1 mutation **is primarily caused by a survival defect of the erythroid progenitors and differentiating erythroblasts**. The study was published in ***Haematologica*** (Attachment 1).

2.1.2 The effect of DMT1 deficiency on mature erythrocytes (Attachment 2)

In further studies we focused on mature DMT1-mutant erythrocytes. It is known that nutritional iron deficiency and certain types of congenital hypochromic anemia are associated with increased levels of ROS and shortened life span of erythrocytes that can be at least partially attributed to a programmed cell death of erythrocytes, so called eryptosis (Lang et al., 2005; Lang et al., 2012).

Eryptosis is characterized by cell shrinkage, cell membrane blebbing, activation of proteases, and cell membrane phospholipid scrambling. Erythrocytes with exposed phosphatidylserines at the outer membrane surface are recognized by macrophages, ingested, and degraded. Eryptosis is triggered by oxidative stress, hyperosmotic shock, and glucose depletion likely via activation of divalent calcium cation (Ca^{2+}) channels leading to an increase in the concentration of cytosolic Ca^{2+} (Duranton et al., 2002; Lang et al., 2003).

We observed an accelerated clearance of DMT1-mutant erythrocytes from circulating blood when compared to wt erythrocytes (Fig. 1).

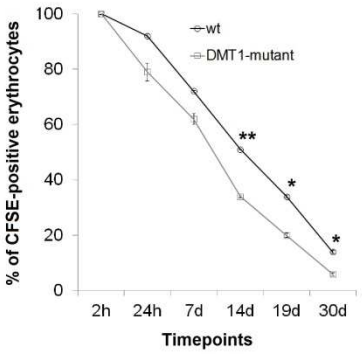


Figure 1. DMT1-mutant erythrocytes show accelarated *in vivo* clearance compared to wild-type (wt) erythrocytes. The percentage of CFSE-labeled erythrocytes was measured by flow cytometry at indicated time points post injection. Values are given as % of CFSE-labeled erythrocytes determined two hours after the injection into the wt mice. h - hours; d - days; **P<0.01, *P<0.05

In vitro, DMT1-mutant erythrocytes showed significantly increased Annexin V binding after exposure to hyperosmotic shock and glucose depletion. This indicated increased phosphatidylserine exposure on the membrane of DMT1-mutant erythrocytes and suggested possible involvement of eryptosis in the shortened life span of DMT1-mutant erythrocytes. It is known that eryptosis is triggered by cytosolic Ca^{2+} activity. Interestingly, a study by Xu et al. in 2004 suggested that the G185R mutation in DMT1 leads to a gain of a constitutive Ca^{2+} permeability. In agreement, we detected significantly

higher content of intracellular Ca^{2+} in DMT1-mutant erythrocytes than in wt erythrocytes (Fig. 2). Besides increased Ca^{2+} we also revealed enhanced accumulation of ROS (Fig. 2).

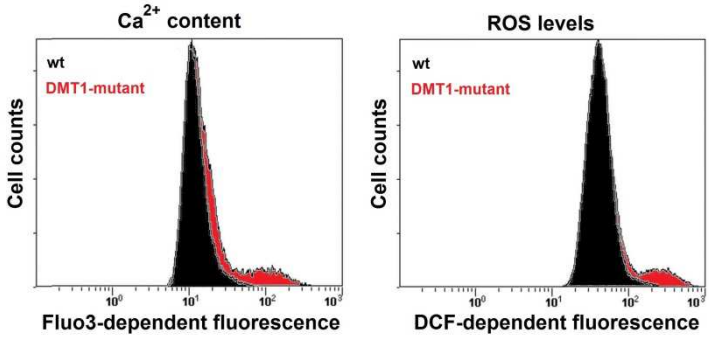


Figure 2. DMT1-mutant erythrocytes have higher content of intracellular Ca^{2+} and increased levels of ROS. Representative histograms of Ca^{2+}-dependent fluorescence and ROS-dependent fluorescence are shown.

Surprisingly, the activity of antioxidant enzymes (glutathione peroxidase, catalase, methemoglobin reductase, glutathione reductase, and glucose-6-phosphate dehydrogenase) measured in erythrocyte lysate was significantly higher for DMT1-mutant erythrocytes than for wt erythrocytes (Table 1). In addition, the expression of *Foxo3A*, a transcription factor responsible for the upregulation of the anti-oxidative defense enzymes (Bakker et al., 2007), was significantly elevated in DMT1-mutant reticulocytes when compared to the wt controls, along with other selected markers of augmented hypoxia sensing. These data suggested that ROS exaggerate anti-oxidative defense in DMT1-mutant erythrocytes but the buffering capacity of this defense is insufficient to eliminate ROS effectively. We proposed that tissue hypoxia may limit the extent of anti-oxidative capacity in DMT1-mutant red blood cells.

	GPx [IU/g Hb]	**MtHbR** [IU/g Hb]	**GR** [IU/g Hb]	**G6PD** [IU/g Hb]	**CAT** [IU/g Hb]
wt	12.0 ± 0.6	27.2 ± 1.0	5.9 ± 0.9	17.8 ± 0.5	26666 ± 5941
DMT1-mutant	18.1 ± 1.1*	50.7 ± 0.9***	14.0 ± 0.5***	32.1 ± 0.8**	49639 ± 7519*

Table 1. The activity of antioxidant enzymes is increased in lysates of DMT1-mutant erythrocytes compared to wt erythrocytes. Enzyme activity is expressed in IU/g hemoglobin (Hb). GPx - glutathione peroxidase, MtHbR - methemoglobin reductase, GR - glutathione reductase, G6PD - glucose-6-phosphate dehydrogenase, CAT - catalase); ***P<0.001, **P<0.01, *P<0.05

We also observed abnormalities in anaerobic glycolysis, another metabolic pathway essential for erythrocytes and responsible for the production of ATP. Selected enzymes of

this pathway (hexokinase, pyruvate kinase, and glucose-phosphate isomerase) showed increased activity in DMT1-mutant erythrocytes in comparison with wt erythrocyte (Table 2). Despite this hyperactivation HPLC-MS/MS measurements for ATP and ADP revealed reduced levels of ATP and consequently reduced ATP/ADP ration in DMT1-mutant erythrocytes (1.7±0.5) when compared to wt erythrocytes (2.8±0.6). This result suggested increased demand for ATP in DMT1-mutant erythrocytes.

	HK [IU/g Hb]	**PK** [IU/g Hb]	**GPI** [IU/g Hb]
wt	2.8 ± 0.1	6.8 ± 0.3	35.4 ± 1.0
DMT1-mutant	6.9 ± 0.2***	16.3 ± 0.5***	113.5 ± 2.1***

Table 2. The activity of enzymes of anaerobic glycolysis is increased in lysates of DMT1-mutant erythrocytes compared to wt erythrocytes.

Enzyme activity is expressed in IU/g hemoglobin (Hb). HK - hexokinase, PK - pyruvate kinase, GPI - glucose-phosphate isomerase; ***P<0.001

In conclusions, this study revealed that **DMT1-deficiency negatively affects erythrocyte metabolism and reduces their capacity to cope with stress**. Increased content of cytosolic calcium, insufficient anti-oxidative defense, and reduced levels of ATP shorten the life span of mature DMT1-mutant erythrocytes. **We proposed that this erythrocyte defect is a contributing factor in the pathophysiology of anemia caused by DMT1 mutations.** The work was published in ***Cell Physiol Biochem.*** (Attachment 2).

2.2 Disordered iron homeostasis in congenital defects of erythropoiesis

Analyses of diverse erythroid and iron metabolism defects have shown that disrupted erythropoiesis negatively affects iron homeostasis and vice versa. The molecular pathophysiology involves disordered production of hepcidin; the key molecule inhibiting iron absorption, mobilization, and recycling (Ganz and Nemeth, 2011).

For example, β-thalassemia (Nemeth, 2010) and congenital dyserythropoietic anemia (Shalev et al., 2013) are associated with ineffective erythropoiesis, which causes hepcidin suppression. As a result, iron absorption and recycling are augmented, leading to the development of secondary iron overload independently of transfusions (Fig. 1).

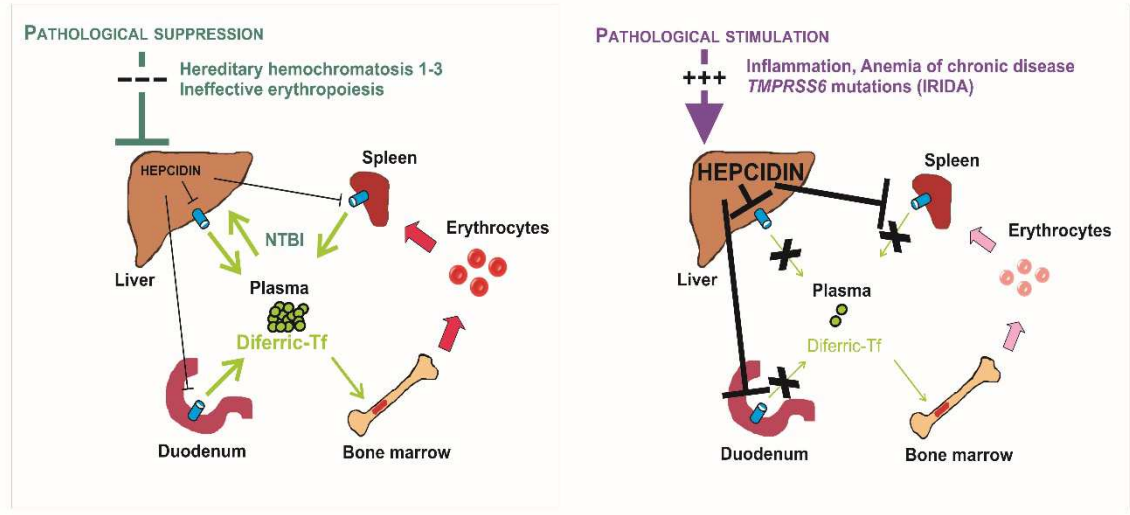


Figure 1. Role of hepcidin in diseases. Pathological suppression of hepcidin in hereditary hemochromatosis and in response to ineffective erythropoiesis leads to increased iron levels in the plasma (differic Tf) and concomitant increase of NTBI resulting in iron overload. Pathological stimulation of hepcidin in response to inflammation or due to a genetic defect in *TMPRSS6* (coding for the negative hepcidin regulator matriptase 2) leads to the development of an iron-restrictive state and anemia. Adapted and modified form Ganz and Nemeth, 2011.

On the other hand, increased plasma hepcidin, due to pathological IL-6 stimulation (Nemeth et al., 2004b; Nemeth and Ganz, 2014) or inherited *TMPRSS6* mutations (Finberg et al., 2008), leads to reduced availability of iron for erythropoiesis and development of anemia in inflammatory disorders and IRIDA, respectively (Fig. 1).

Four original manuscripts and three review articles are presented in this section. The following commentary summarizes the most important data which are a part of the original manuscripts. The review articles (Attachments 7, 8 and 9) will not be commented as they represent the state-of-the-art on iron metabolism and iron homeostasis defects and served (in part) as a background for the Introduction chapter.

1. Pospíšilová D, Holub P, Houda J, Ludíková B, Mojzíková R, Pospíšilová P, Židová Z, Kapraľová K, **Horváthová M**, Hajdúch M, Džubák P. Význam stanovení hladiny hepcidinu v diagnostice vybraných typů anémií v dětském věku. [Significance of hepcidin level assessment in the diagnosis of selected types of anaemia in childhood], article in Czech. ***Transfuze hematol. dnes***. 2012, 18, 58-65. (Attachment 3)

2. Mojzikova R, Koralkova P, Holub D, Zidova Z, Pospisilova D, Cermak J, Striezencova Laluhova Z, Indrak K, Sukova M, Partschova M, Kucerova J, **Horvathova M**, Divoky V. Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinaemia associated with a novel frameshift deletion in the PKLR gene (p.Arg518fs), and low hepcidin to ferritin ratios. ***Br J Haematol***. 2014; 165:556-63. (Attachment 4)

3. Pospisilova D, Holub D, Zidova Z, Sulovska L, Houda J, Mihal V, Hadacova I, Radova L, Dzubak P, Hajduch M, Divoky V, **Horvathova M**. Hepcidin levels in Diamond-Blackfan anemia reflect erythropoietic activity and transfusion dependency. ***Haematologica***. 2014; 99: e118-21. (Attachment 5)

4. Sulovska L, Holub D, Zidova Z, Divoka M, Hajduch M, Mihal V, Vrbkova J, **Horvathova M**, Pospisilova D. Characterization of iron metabolism and erythropoiesis in erythrocyte membrane defects and thalassemia traits. ***Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub***. 2016; 160: 231-7. (Attachment 6)

5. **Horvathova M**, Ponka P, Divoky V. Molecular basis of hereditary iron homeostasis defects. ***Hematology***. 2010; 15: 96-111. (Attachment7)

6. **Horváthová M**, Pospíšilová D. Nové poznatky o homeostáze železa a jejich důsledky pro klinickou praxi. [Advances in iron homeostasis; implications for clinical practice], article in Czech. ***Postgrad Med***. 2010; 6: 676-81. (Attachment 8)

7. Houda J, Pospíšilová D, **Horváthová M**. Úloha hepcidinu v regulaci metabolismu železa. [The role of hepcidin in iron metabolism], article in Czech. ***Čes-slov Pediat.*** 2014; 69: 301-12. (Attachment 9)

2.2.1 Hepcidin in selected types of anemia (Attachment 3)

In a pilot study, published in a Czech peer review journal (***Transfuze hematol. dnes***), we simply evaluated hepcidin levels and their diagnostic contribution in 33 pediatric patients with selected types of anemia including IDA (n=10), PK deficiency (n=5), DBA (n=6), and anemia in inflammatory bowel disease (IBD, n=12). Healthy age-matched children undergoing minor surgery served as normal controls for hepcidin measurents. As expected, hepcidin was significantly low in IDA reflecting iron-restricted erythropoiesis and diminished iron stores. PK-deficient patients showed also significantly lower hepcidin than controls, likely reflecting accelerated ineffective erythropoiesis. On the other hand, hepcidin levels were significantly increased in severely anemic transfusion-dependent DBA patients compared to controls; repeated transfusions and the absence of erythroid-derived hepcidin suppressor due to diminished bone marrow erythropoiesis emerged as potential causes. Finally, the hepcidin levels in IBD patients were comparable with the controls and likely reflected higher iron requirement for erythropoiesis in pediatric age. It is possible that the stimulating effect of iron stores and inflammation on hepcidin production in IBD becomes apparent over time.

In conclusion, we confirmed that **hepcidin levels differentiate different types of anemia and may assist in differential diagnosis and eventually in treatment decisions.**

This initial study led us to elucidate the erythroid regulation of hepcidin production and the crosstalk between erythropoiesis and iron metabolism in more details. In subsequent detailed studies, we aimed to analyze the imbalanced iron metabolism in selected congenital defects of erythropoiesis including PK deficiency, DBA, erythrocyte membrane defects, and thalassemia traits.

2.2.2 Iron metabolism in pyruvate kinase deficiency (Attachment 4)

Erythrocyte PK deficiency is the most common cause of non-spherocytic hemolytic anemia in central and northern Europe. It is characterized by chronic hemolysis, ineffective erythropoiesis, and requirements for blood transfusion in most cases. PK-deficient patients may develop secondary iron overload (Zanella et al., 1993). Although repeated blood transfusions were considered to be the major cause, iron accumulation also affects non-transfused PK-deficient patients. The pathogenesis of iron overload appears to be multifactorial, involving chronic hemolysis, ineffective erythropoiesis, splenectomy, and eventually coinheritance of hereditary hemochromatosis.

We studied a cohort of eleven patients with PK deficiency from ten unrelated families. All patients suffered from mild to severe hemolytic anemia (with Hb levels ranging from 65 to 121 g/l) and hyperbilirubinemia. All but one of these patients had increased reticulocyte counts. All transfusion-dependent patients presented iron overload (increased ferritin and TSAT); one patient has developed hyperferritinemia independently of blood transfusions consistent with findings by Zanella et al. (1993).

The co-inheritance of HFE mutations was detected in four patients; three of these patients were children. Therefore it was difficult to assess the contribution of abnormal HFE genotype to the excessive iron accumulation as HFE-related hemochromatosis is an adult-onset disease (Pietrangello, 2010). Nevertheless, one pediatric patient, a compound heterozygote for HFE mutations (H63D/C282Y), was the only child with normal ferritin levels and therefore it is likely that HFE genotype does not play a major role in determining iron loading in PK-deficient patients at pediatric age. However, a follow up of these patients may be important due to potential higher risk of iron overload in adulthood (Zannella et al, 2001).

Although the hepcidin levels in PK-deficient patients were insignificantly reduced compared to healthy age-matched controls, the difference in hepcidin/ferritin ratio reached statistical significance (median, 0.06 for PK deficiency; median, 0.35 for controls) (Fig. 1).

This ratio represents more accurate estimation of proper hepcidin production with respect to iron loading and clearly confirms suppression of hepcidin by an erythroid signal that overrides iron loading-induced signaling.

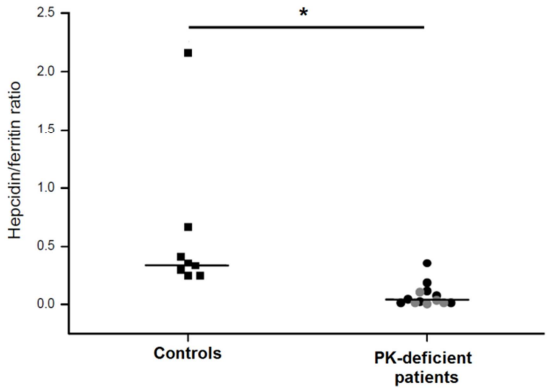


Figure 1. The hepcidin/ferritin ratio is significantly lower in PK-deficient patients compared to healthy controls. Grey dots indicate the hepcidin/ferritin ratio calculated from five repeated measurements for the same patient. *P<0.05

Similar results were published for β-thalassemia intermedia where the inadequate hepcidin attenuation was attributed to ineffective erythropoiesis (Origa et al, 2007) and increased levels of GDF15 (Tanno et al, 2007). The elevation of GDF15 in our PK-deficient cohort (median 905.5 ng/l *vs* median in controls 223 ng/l) was considerably lower than that reported for β-thalassemia. No correlation between GDF15 and hepcidin or hepcidin/ferritin ratio indicated that GDF15 does not suppress hepcidin expression in PK deficiency and therefore GDF15 can only be used as a marker of accelerated ineffective erythropoiesis (Tanno et al, 2010).

Similarly to elevated GDF15 we also found increased levels of EPO, which again did not correlate with hepcidin or hepcidin/ferritin ratio. This supports conclusions of others (Pak et al, 2006; Vokurka et al, 2006) that EPO by itself is an indirect suppressor of hepcidin.

In conclusion, **we detected inappropriately low levels of hepcidin with respect to iron loading in all PK-deficient patients with increased ferritin, confirming the predominant effect of accelerated erythropoiesis on hepcidin production**. **The lack of negative correlation between hepcidin and GDF15 or EPO** indicated the existence of another erythroid regulator of hepcidin synthesis in PK deficiency. Our results were published in ***Br J Haematol.*** (Attachment 4).

2.2.3 Deregulated iron metabolism in Diamond-Blackfan anemia (Attachment 5)

DBA is a rare congenital red cell aplasia resulting from haploinsufficiency of ribosomal proteins (RP) in 49-71% of cases (Boria et al., 2010). It is characterized by variable degree of reduced erythropoietic activity in the bone marrow and diverse disease severity. Approximately one-third of patients develop transfusion-acquired iron overload (Vlachos et al., 2008).

Our cohort consisted of twenty-five DBA patients from the Czech National DBA Registry (Pospisilova et al., 2012). The patients were grouped based on the type of treatment into: 1) patients on regular transfusions with or without chelation therapy (TDP, n=12); 2) patients treated with steroids (SP, n=4); 3) patients in disease remission without treatment (RP, n=7); and 4) patients treated with corticosteroids and occasional transfusions (S,T; n=2) (Table 1).

Table 1. Parameters of erythropoiesis and iron metabolism in DBA patients.

	TDP (n=12)	RP (n=7)	SP (n=4)	S,T* (n=2)	Reference range
Age (years)	7.6 (1.0-27.9)	26.9 (13.3-35.8)	32.6 (27.0-42.9)	25.8 25.9	–
Hepcidin (ng/mL)	341.5 (165.1-572.6)	72.1 (29.5-153.3)	24.7 (8.5-100.1)	173.9 144.9	27.6 (13.1-104.8)
Ferritin (ng/mL)	1290 (343-3747)	177 (42-1079)	188 (44-637)	3150 868	22-275
Hepcidin/ferritin ratio	0.240 (0.09-1.35)	0.222 (0.09-1.04)	0.151 (0.10-0.19)	0.06 0.17	0.35 (0.2-2.2)
Fe (μmol/L)	41.5 (35.8-59.0)	20.0 (10.0-30.6)	21 (14.7-33.4)	39.6 43.2	7.2-29
TSAT (%)	91 (61-100)	38 (25-55)	64 (63-65)	93 94	21-48
LIC (mg/g d.w.)	7.3# (4.6-16.3)	*ND*	*ND*	*ND*	0.3-1.4
GDF15 (pg/mL)	676.2 (352.1-1694.8)	927.0 (556.3-2824.8)	1127.6 (408.8-2190.8)	4221.5 3497.1	223 (166-344)
EB (%)	0.8 (0.4-8.2)	21.4 (8.4-34.0)	20.4 (16.8-33.4)	10.6 20.8	15.0-25.0
EPO (IU/L)	2452 (837-6476)	137 (20-1913)	441 (287-500)	615 4000	4.3-29.0
sTfR (mg/L)	*ND*† <0.5-1.2	2.3 (1.3-3.2)	2.4 (1.8-2.8)	1.4 1.4	1.9-4.4

Table legend: Values are shown as medians and the full range of variation. TDP: transfusion-dependent patients; RP: patients in disease remission; SP: patients treated with steroids; S,T: patient on steroids and occasional transfusion; Fe: serum iron; TSAT: transferrin saturation; LIC: liver iron concentration, d.w.: dry weight; GDF15: growth differentiation factor 15; EB: erythroblasts in the bone marrow; EPO: serum erythropoietin; sTfR: soluble transferrin receptor; ND: not determined. *: individual values are shown; #: values available for 5 patients; †: median could not be calculated as 9 of 12 patients had sTfR bellow the limit of detection (less than 0.5 mg/L). Pre-transfusion samples were collected for TDP.

Severely suppressed erythropoiesis was confirmed in all transfusion-dependent DBA patients. They had reduced number of erythroblasts in the bone marrow and markedly decreased sTfR (often under the lower limit of detection). Analysis of iron parameters showed increased serum iron, TSAT, and high ferritin levels suggesting defective erythroid utilization of transferrin-bound iron (Table 1). Consistently, liver biopsy revealed markedly elevated liver iron concentration (LIC) and massive iron stores in both Kupffer cells and hepatocytes that can likely be attributed to non-effective erythrocyte-derived iron recycling and increased NTBI uptake, respectively.

The other two groups of DBA patients, patients in disease remission or patients treated with steroids, showed near normal or slightly higher number of bone marrow erythroblasts and normal levels of sTfR suggesting improved erythropoiesis. These patients had slightly increased or even normal levels of ferritin and near normal serum iron and TSAT (Table 1). This indicates that improved erythropoietic activity leads to improved iron utilization and consequently to normalization of ferritin levels.

The measurements of hepcidin revealed that DBA patients on regular transfusions showed significantly elevated hepcidin similar to what was published for transfusion-dependent beta-thalassemia major patients (Origa et al., 2007). In contrast, DBA patients in remission or treated with steroids had hepcidin levels comparable to controls (Table 1).

The two patients on corticosteroids and occasional transfusions were evaluated independently. They both had normal sTfR, almost normal number of bone marrow erythroblasts, but elevated ferritin as well as hepcidin and reduced hepcidin/ferritin ratio (Table 1). One of these two patients (#24) was found to be a homozygote for the C282Y HFE mutation and had the second highest ferritin associated with relatively low level of hepcidin (Table 1). HFE C282Y homozygotes are known to have very low levels of hepcidin due to the improper stimulation of hepcidin expression (van Dijk et al., 2008). The concomitant occurrence of DBA and abnormal HFE genotype can explain the reduction of hepcidin synthesis in this patient in comparison with other DBA patients with comparable hyperferritinemia. Therefore, this patient was excluded from statistical analyses of hepcidin levels correlations.

As erythropoiesis is known to produce a signal for hepcidin suppression, DBA patients with restored erythropoietic activity are likely to be able to attenuate hepcidin expression and thus increase the iron pool available for improved erythropoiesis. In

agreement, we detected a trend towards lower hepcidin/ferritin ratio in steroids-receiving patients (median 0.151), patient on low-dose steroids and occasional transfusions (0.170) or patients in remission (median 0.222) when compared to transfusion-dependent patients (median 0.240) and healthy controls (median 0.35) (Table 1). These results indicate that the bone marrow of transfusion-dependent DBA patients is probably not releasing the putative erythroid suppressor of hepcidin production, making it different from beta-thalassemia major with known contribution of erythropoiesis-related hepcidin attenuation (Pasricha et al., 2013).

Serum EPO was substantially elevated above the upper limit in most of DBA patients (Table 1). When EPO was compared between differently treated patients, transfusion-dependent patients showed the highest levels of EPO, followed by the steroids-receiving group. The lowest levels were detected in patients who are currently in disease remission, with three patients having their EPO within the normal range. A negative correlation between EPO and the number of erythroblasts in the bone marrow confirmed that EPO (Fig. 1) is stimulated in response to suppressed erythropoiesis and hypoxia.

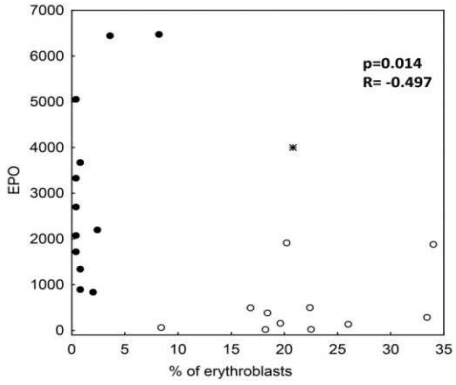


Figure 1. Serum EPO levels in DBA patients negatively correlate with the number of bone marrow erythroblasts (expressed in %). closed circles - transfusion-dependent patients, open circles - patients on steroids or in remission, asterisk - one patient on a low dose of steroids and occasional transfusions; p – statistical significance; R - Spearman coefficient.

Similar to EPO, we also detected significantly increased levels of GDF15 for the whole DBA cohort as well as for the differently treated groups when compared to normal controls (Table 1). The levels of GDF15 in DBA were lower than in thalassemia patients and more comparable with those detected in patients with PK deficiency (Mojzikova et al., 2014).

As GDF15 has been proposed to be secreted by dying erythroblasts (Tanno et al., 2010) and increased apoptosis of erythroid cells is a characteristic feature of DBA (Boria et al., 2010) we evaluated the extent of apoptosis in the bone marrow of two DBA patients - one in remission and the other one treated with steroids and occasional transfusions.

The TUNEL assay on trephine biopsy and co-staining with an erythroid marker Glycophorin A (GlyA) showed a higher number of erythroblasts undergoing apoptosis (GlyA+/TUNEL+) when compared to the control bone marrow sample (Fig. 2). Thus, the increased rate of apoptosis observed in DBA erythroblasts may contribute to the elevated levels of GDF15.

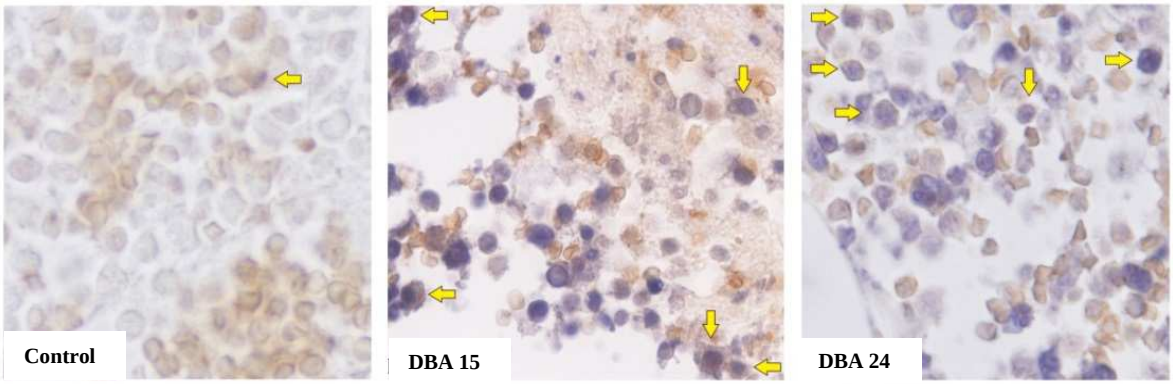


Figure 2. Increased apoptosis of bone marrow erythroblasts in two DBA patients compared to healthy control. TUNEL assay shows significant elevation in the number of erythroid Glycophorin A+ cells (brown color) undergoing apoptosis (TUNEL+, dark purple color) in the bone marrow. The arrows indicate Glycophorin A+/TUNEL+ cells.

The limited number of samples precluded any correlation analysis between the rate of apoptosis and GDF15 levels. The presence of apoptotic erythroblasts in the bone marrow of DBA patient in remission suggests that although erythropoiesis is seemingly restored, a considerable degree of cell death is present. Analysis of more patients in remission with different RP mutations is needed to address whether this finding is mutation-specific or not.

Finally, we aimed to establish which of the aforementioned signals/markers contribute to the regulation of hepcidin synthesis in DBA. Hepcidin positively correlated with ferritin (Fig. 3A), reflecting hepcidin stimulation by the patients' iron overload. An inverse correlation between the percentage of bone marrow erythroblasts and hepcidin levels (Fig. 3B) is consistent with the negative regulation of hepcidin synthesis by

erythropoietic activity. A positive correlation between hepcidin and EPO (Fig. 3C), which should have been in fact negative, confirms that EPO is not able to suppress hepcidin synthesis directly (Pak et al, 2006; Vokurka et al, 2006). Furthermore, neither hepcidin nor hepcidin/ferritin ratio correlated with GDF15 indicating that GDF15 is not playing a hepcidin-regulatory role in DBA.

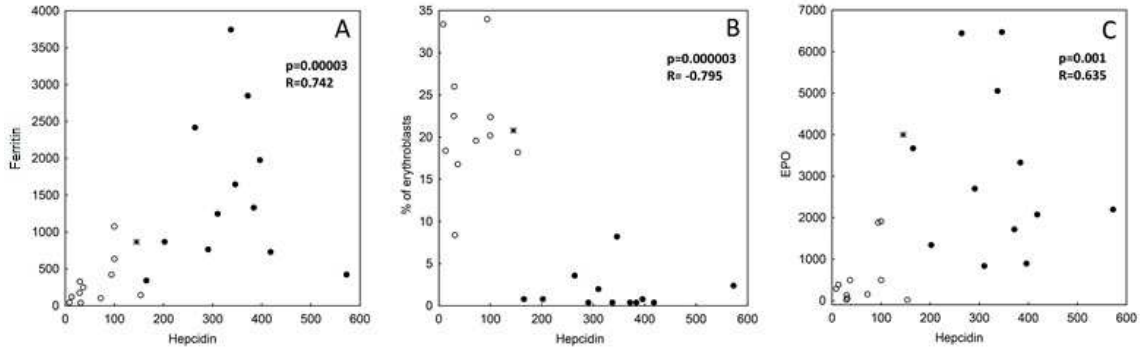


Figure 3. Hepcidin levels show the following correlations: A) positive with ferritin, B) negative with the number of erythroblasts in the bone marrow, and **C) positive with serum EPO.** Closed circles - transfusion-dependent patients, open circles - patients on steroids or in remission, asterisk - one patient on a low dose of steroids and occasional transfusions; p – statistical significance; R - Spearman coefficient

In conclusion, our study suggested that **diverse levels of hepcidin and iron overload in DBA patients reflect transfusion dependency and variable erythropoietic activity in their bone marrow**. In conditions of severely suppressed erythropoiesis, hepcidin production is not attenuated. Our results also confirmed that **hepcidin suppression by EPO requires active erythropoiesis in the bone marrow**. This study was published in ***Haematologica*** (Attachment 5).

2.2.4 Erythropoiesis and iron metabolism in erythrocyte membrane defects and thalassemia traits (Attachment 6)

Recent publications showed that besides thalassemia intermedia and major also carriers of β- or α-thalassemia alleles have altered parameters of iron metabolism and erythropoiesis despite the absence of clinical symptoms (Guimarães et al., 2015; Jones et al., 2015). Because thalassemia minor is the most common cause of congenital hypochromic microcytic anemia in the Czech Republic, we decided to investigate the imbalanced iron homeostasis in the Czech pediatric thalassemia carriers. Pediatric patients with erythrocyte membrane defect were also included in this study as this condition is the most common cause of inherited hemolytic anemia in the Czech population and the knowledge of the interconnection between disrupted erythropoiesis and iron metabolism in erythrocyte membrane defects is limited.

Hereditary disorders of the erythrocyte membrane are clinically and genetically heterogeneous and chacaterized by the formation of abnormally shaped erythrocytes, *i.e.* spherical for HS or elliptical for HE. Patients with HE or HS may be asymptomatic or present with varying degree of hemolytic anemia, increased reticulocyte count and serum bilirubin, and splenomegaly. The most severe cases are treated with blood transfusions, which may eventually lead to iron overload (An and Mohandas, 2008).

The pathophysiology and clinical symptoms of thalassemia are heterogeneous and were discussed in details in Chapter ***1.3.1.3 α- and β-thalassemia***. β-thalassemia minor (β-thalassemia trait) and α-thalassemia minor (α-thalassemia trait) represent the mildest and usually clinically asymptomatic form with hypochromic microcytic erythrocytes and compensatory erythrocytosis.

The cohort analyzed in this study consisted of 20 pediatric patients with erythrocyte membrane defect (HS and HE), 13 pediatric subjects with thalassemia minor (thalassemia carriers), and 1 patient with thalassemia major (TM) (age 7 years), an immigrant from Moldova. The TM patient served as a positive control for disrupted erythropoiesis and iron metabolism including inappropriately low hepcidin. The parameters of this patient were comparable with the data published on TM patients in the literature (Nemeth, 2010; Pasricha et al., 2013) .

The anemia in erythrocytes membrane defect group was hyperchromic, milder than in thalassemia carriers, and accompanied by reticulocytosis. In accordance with recent

publications (Guimarães et al., 2015; Jones et al., 2015) also pediatric thalassemia carriers had altered red blood cell parameters. Elevated sTfR and GDF15 indicated increased, but ineffective bone marrow erythropoiesis for both groups. All thalassemia carriers had serum iron and ferritin within the normal range, but the hepcidin levels and hepcidin/ferritin ratio were reduced. Patients with erythrocyte membrane defect showed normal serum Fe, but significantly elevated ferritin and reduced hepcidin and hepcidin/ferritin ratio compared to the controls. These results suggest an inappropriate attenuation of hepcidin synthesis. The direct signals remain to be identified.

Recently, it has been suggested that a ratio of (hepcidin/ferritin) to sTfR differentiates adult thalassemia carriers from healthy controls and reflects the severity of anemia. Indeed this ratio was reduced in both thalassemia carriers and erythrocyte membrane defects compared to controls confirming disordered interaction between iron metabolism and erythropoiesis. Moreover both patients group dislocate from controls on the graph plotting sTfR against hepcidin/ferritin (Fig. 1). The erythrocyte membrane defects are shifted more to the top right than thalassemia carriers indicating more pronounced disbalance between erythropoiesis and iron metabolism.

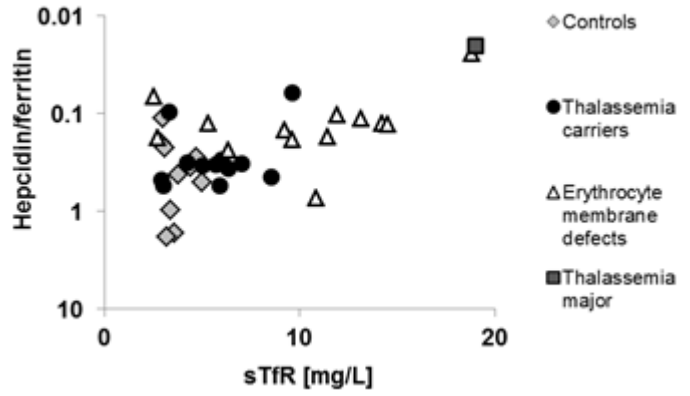


Figure 1. Differentiation of patients based on the plot of hepcidin/ferritin ratio against sTfR15. Patients with erythrocyte membrane defect (n=13), thalassemia carriers (n=12) and thalassemia major (TM, n=1) dislocate from healthy controls (n=9).

It is important to mention that the negative effect on erythropoiesis is also likely dependent on the molecular identity of the diseases causing alleles as these alleles may differ in their severity and thus be responsible for interindividual differences among the carriers; this may be particulary important for the thalassemia group.

We can summarize that **pediatric patients with erythrocyte membrane defect** and **thalassemia carriers showed alterations in erythropoiesis and iron metabolism, that are nicely reflected by reduced (hepcidin/ferritin)/sTfR ratio**. The regulation of iron homeostasis appears to be more disbalanced in erythrocyte membrane defects than in thalassemia carriers, but to a lesser extent than in thalassemia major. We suppose that **patients with erythrocyte membrane defects** and **thalassemia carriers may be more susceptible to iron overload than the general population**. This study, showing for the first time in the literature, disturbed balance between erythropoiesis and iron metabolism in erythrocyte membrane defects (HS and HE) was published in ***Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.*** (Attachment 6).

2.3 Molecular pathophysiology of inherited erythrocytoses

The molecular pathophysiology of inherited erythrocytoses (or polycythemias) includes abnormalities in the key pathways regulating red blood cells formation: EPOR/JAK2/STAT5 pathway and HIF signaling pathway or defects causing increased affinity of hemoglobin for oxygen (Prchal, 2010). Despite an intense research the causative mutations remain, in the vast majority of congenital erythrocytoses, unknown. It is believed that these alterations affect other molecules involved in the pathways regulating erythropoiesis.

Three original manuscripts and one article which summarizes the current state of the understanding of the topic together with our own results are presented in this section.

1. Kučerová J, **Horváthová M**, Pospíšilová D, Divoký V. Vrozené polycytemie. [Congenital polycythemias], article in Czech. ***Transfuze Hematol. dnes***. 2009; 15: 216-22. (Attachemnt 10)

2. Kapralova K, **Horvathova M**, Pecquet C, Fialova Kucerova J, Pospisilova D, Leroy E, Kralova B, Milosevic Feenstra JD, Schischlik F, Kralovics R, Constantinescu SN, Divoky V. Cooperation of germline JAK2 mutations E846D and R1063H in hereditary erythrocytosis with megakaryocytic atypia. ***Blood***. 2016 Jul 7. pii: blood-2016-02-698951. [Epub ahead of print] (Attachemnt 11)

3. Kapralova K, Lanikova L, Lorenzo F, Song J, **Horvathova M**, Divoky V, Prchal JT. RUNX1 and NF-E2 upregulation is not specific for MPNs, but is seen in polycythemic disorders with augmented HIF signaling. ***Blood***. 2014; 123: 391-4. (Attachemnt 12)

4. Divoky V, Song J, **Horvathova M**, Kralova B, Votavova H, Prchal JT, Yoon D. Delayed hemoglobin switching and perinatal neocytolysis in mice with gain-of-function erythropoietin receptor. ***J Mol Med (Berl).*** 2016; 94: 597-608. (Attachemnt 13)

2.3.1 Cellular and genetic characterization of patients with suspected erythrocytosis (Attachment 10)

Biochemical, cellular, and molecular biology tests are important for the evaluation of polycythemia type and for differential diagnostics (Fig. 12 in Chapter *1.3.2 Polycythemia*). Some of these tests has successfully been established in our laboratories and include the *in vitro* sensitivity test of erythroid progenitors to exogenously added EPO and targeted mutational analysis of known genes associated with congenital or acquired polycythemia.

Our publication on congenital polycythemias in a Czech peer review journal (***Transfuze Hematol. dnes***, Attachment 10) was presented as an overview of the literature with the addition of our personal experience with the diagnostics of polycythemia states. We identified a previously published *EPOR* mutation (5967insT) in two boys (Table 1, patients #1 and #2) in a cohort of seven patients with suspected erythrocytosis and hypersensitivity of erythroid progenitors to EPO (Table 1).

Table 1. Erytrocytoses with EPO hypersensitivity.

Patient	EPO [IU/l] (4.3-29)	EPO hypersen.	Mutational analysis					
			JAK2	EPOR	LNK	HIF1A, 2A	VHL	PHD2
1	12	yes	*ND*	**5967insT** het	*ND*	*ND*	*ND*	*ND*
2	5	yes	*ND*	**5967insT** het	*ND*	*ND*	*ND*	*ND*
3	3.1	yes	neg.	neg.	neg.	neg.	neg.	neg.
4	2.4	yes	neg.	neg.	neg.	neg.	neg.	neg.
5	7.3	yes	*ND*	*ND*	*ND*	**G537R HIF2A** het	*ND*	*ND*
6	10.4	yes	*ND*	*ND*	*ND*	**G537R HIF2A** het	*ND*	*ND*
7	7	yes	**E846D** het **R1063H** het	neg.	neg.	neg.	neg.	neg.

Table legend: EPO hypersen. – EPO hypersensitivity, *ND* – not done, neg. – negative result (no mutation detected), het - heterozygote.

In the years following the publication additional abnormalities were identified in the cohort. The known G537R substitution in HIF2A was found in two unrelated children (Table 1, patients #5 and #6). These two patients together with one EPOR-mutant patient (#2) were included in a collaborative study with J.T. Prchal aiming to define the role of increased RUNX1 and NF-E2 expression in the hypersensitivity of erythroid progenitors (for details see the comment on Attachment 12, Chapter 2.3.3).

Highly important from the original cohort is patient #7 (Table 1) in whom **two germline heterozygous mutations in JAK2 (E846D and R1063H)** were detected. Detailed analysis of this patient and his family resulted in a postulation of a new model for the development of **hereditary erythrocytosis with megakaryocytic atypia**. The study is discussed in details in the following Chapter 2.3.2 and Attachment 11.

Since the original description of the cohort it has been enlarged by seven additional subjects with *in vitro* hypersensitivity of erythroid progenitors to EPO. So far no disease causing mutations were detected in these new patients.

We can conclude that the results obtained on our cohort are consistent with the data published in the literature, where a considerable number of patients is classified as **idiopathic erythrocytosis.**

Based on our analyses we propose that some of these **patients may harbour predisposing mutation/polymorphism and the full disease phenotype becomes apparent in combination with another/other inherited or acquired interacting lession(s)**.

2.3.2 Cooperating JAK2 germline mutations in hereditary erythrocytosis with megakaryocytic atypia (Attachment 11)

The role of somatic JAK2 mutations in clonal MPN is well established (Tefferi et al, 2009). Recently, germline JAK2 mutations were associated with polyclonal hereditary thrombocytosis and triple negative MPN (Etheridge et al., 2014, Marty et al., 2014; Mead et al., 2013; Milosevic Feenstra et al., 2016).

Here, we studied a patient exhibiting congenital erythrocytosis with plethora and splenomegaly. The analysis of bone marrow showed hypercellularity, erythroid hyperplasia, and abnormal megakaryopoiesis (Fig. 1), partially resembling PV cases with JAK2 exon 12 mutations (Lakey et al., 2010).

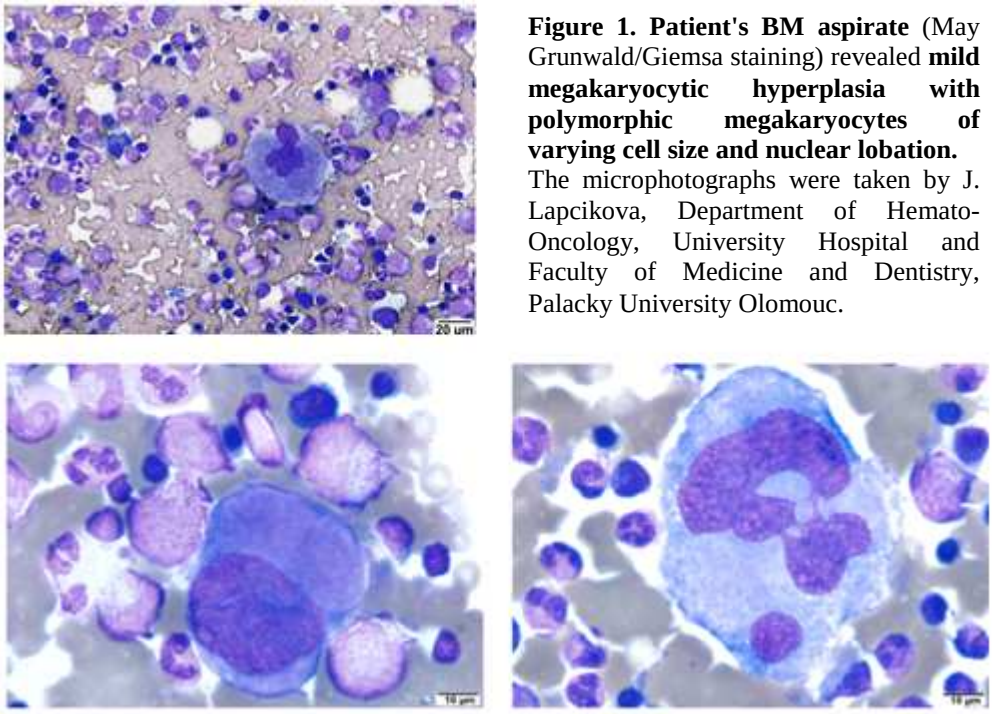
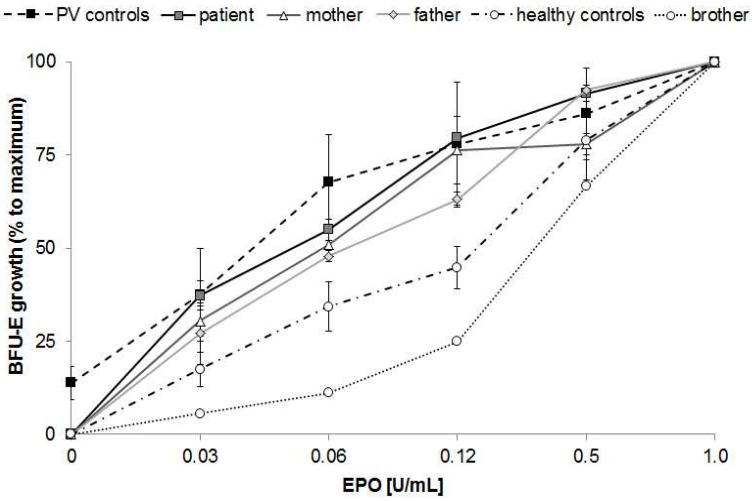


Figure 1. Patient's BM aspirate (May Grunwald/Giemsa staining) revealed **mild megakaryocytic hyperplasia with polymorphic megakaryocytes of varying cell size and nuclear lobation.** The microphotographs were taken by J. Lapcikova, Department of Hemato-Oncology, University Hospital and Faculty of Medicine and Dentistry, Palacky University Olomouc.

Erythroid progenitors from the patient and his parents exhibited *in vitro* hypersensitivity to EPO (Fig. 2), suggesting the hereditary nature of the disease.



Statistical significance of the growth compared to healthy controls

EPO [U/mL]	Patient	Mother	Father	PV
0.0	*NS*	*NS*	*NS*	**0.00002**
0.03	**0.00847**	**0.03445**	*NS*	**0.03115**
0.06	**0.01636**	**0.03039**	*NS*	**0.00698**
0.12	**0.00103**	**0.00496**	**0.011**	**0.00424**
0.5	*NS*	*NS*	*NS*	*NS*

Figure 2. Erythroid progenitors from the patient, his mother, and partially also from his father are hypersensitive to EPO, producing relatively higher number of BFU-E colonies in comparison with healthy control in low EPO concentrations.

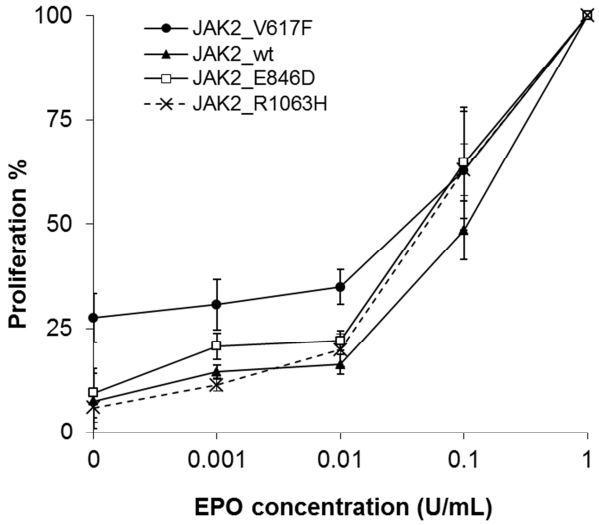
The progenitors of patient's brother showed normal growth. The growth of BFU-E erythroid colonies at the indicated concentrations of EPO is expressed as a percentage of maximum EPO stimulation (represented by EPO concentration of 1 U/mL). Two PV patients, positive for V617F mutation, were used as positive controls for hypersensitivity and formation of EPO independent colonies (EECs). The table on the right side of the figure shows statistical evaluation of the BFU-E colony number at individual concentrations with respect to normal controls.

Combination of targeted mutational analysis and whole exome sequencing revealed an E846D substitution in JAK2 in a heterozygous state in the propositus and his mother and heterozygous substitutions JAK2 R1063H, TET2 P174H, and EPO G84R in the propositus and his father. No somatic variants were found.

The search of the literature suggested that the two germline JAK2 mutations may be the main disease causing events in our patient. This is supported by the fact that the E846D substitution was previously reported in normal-karyotype acute myeloid leukemia (Schnittger et al., 2009) and JEG-3 cancer cell line (Matthews et al., 2010) and the R1063H in three JAK2 V617F-positive PV patients (Levine et al., 2005), as well as in acute myeloid leukemia (Schnittger et al., 2009) and B-cell acute lymphoblastic leukemia cases (Sadras et al., 2015). By screening a large cohort of subjects (n=99) we were able to find the JAK2 E846D substitution in the germline in one V617F-positive/calreticulin-positive MPN patient and JAK2 R1063H in the germline in one V617F-positive PV patient, but not in healthy controls.

We therefore focused on the functional effect of these two JAK2 variants. Using Ba/F3-EPOR JAK2 transfectants we revealed that both E846D and R1063H mutants promote proliferation at low EPO concentrations (Fig. 3) and increase activation of STAT5

(Fig. 4A). Increased activation of STAT5 was also confirmed by immunofluorescence analysis on patient's bone marrow (Fig. 4B).



Statistical significance of transfectants' proliferation compared to JAK2_wt

EPO [U/mL]	E846D	R1063H	V617F
0.1	0.01781	0.00156	0.03868
0.01	0.04079	*ns*	0.00102
0.001	0.04091	*ns*	0.0068
0.0	*ns*	*ns*	4.37E-04

Figure 3. Increased proliferation of BaF3 transfectants expressing JAK2 E846D and R1063H compared to JAK2 wt in media with low concentration of EPO. JAK2 V617F transfectants were used as a positive control.

The percentage of proliferating cells (determined by MTT assay) was calculated as the percentage of the maximum cells growth observed at EPO concentration of 1.0 U/mL. The table on the top right side of the figure shows statistical evaluation of the proliferation for individual transfectants compared to transfectants expressing JAK2 wt.

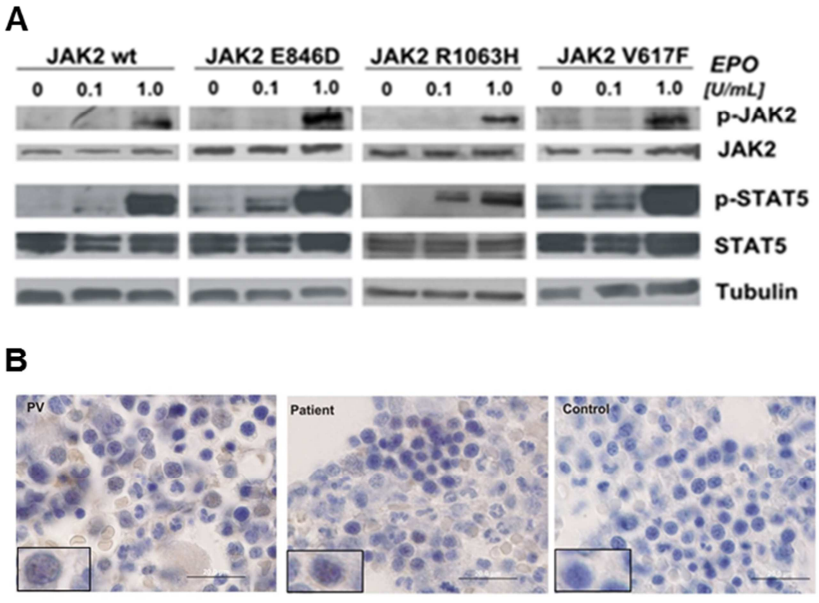


Figure 4. Increased STAT5 activation (p-STAT5) detected A) for JAK2_E846D and JAK2_R1063H transfectants and **B) in patient's bone marrow** (brown nuclear staining). The insets (Panel B) show one cell in detail.

BaF3 cells expressing JAK2 V617F (Panel A) and JAK2 V617F-positive PV patient (Panel B) served as positive controls for constitutive STAT5 activation.

Dual-reporter luciferase assay in γ-2A cells transfected with different JAK2 expressing vectors revealed that both E846D and R1063H weakly hyperactivate

JAK2/STAT5 signaling only in the specific context of EPOR (see the manuscript, Attachment 11).

Analyses on primary erythroid cells of the patient and his parents together with mouse bone marrow cells retrovirally transduced with murine JAK2 expression vectors confirmed that JAK2 E846D increases capacity for erythroid CFU-E and BFU-E colony formation, while JAK2 R1063H increases only formation of CFU-Es (Fig. 5). This result is consistent with the data from other assays (MTT proliferation assay, immunoblot analyses of JAK2 signaling, and luciferase assay) showing that the JAK2 E846D has stronger effects than JAK2 R1063H. Nevertheless, the effect of both studied germline JAK2 variants is much weaker than that of the oncogenic JAK2 V617F, enabling they transmission through the germline.

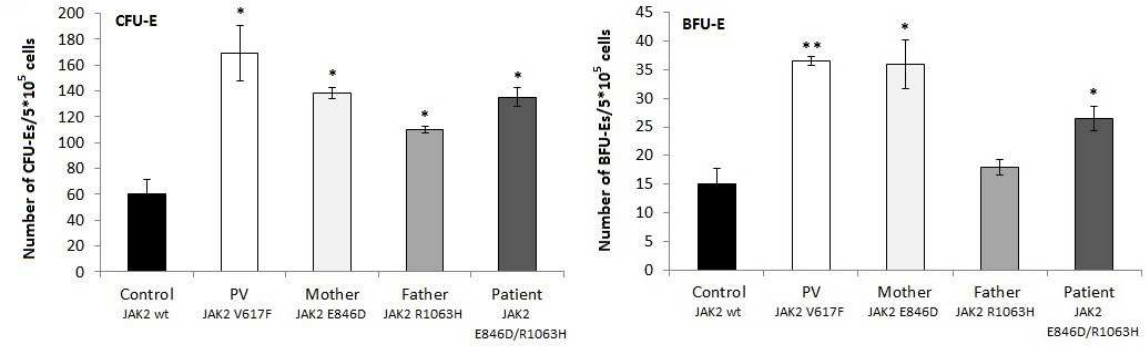


Figure 5. Replating capacity of the primary cells with JAK2 mutations. In secondary EPO-limited cultures we detected: **A) significantly higher number of CFU-E colonies for the PV samples** (JAK2 V617F) and **samples of the patient** (JAK2 E846D/R1063H)**, his mother** (JAK2 E846D) and **father** (JAK2 R1063H). **B) BFU-E colony number was significantly increased for** the **PV samples** (JAK2 V617F) and **samples of the patient** (JAK2 E846D/R1063H) and **his mother** (JAK2 E846D). The number of BFU-E colonies derived from JAK2 R1063H sample of patient's father was comparable to the number of control BFU-Es with wt JAK2. For the results on transduced murine bone marrow cells please see the manuscript.

We propose that **JAK2 E846D predominantly contributes to congenital erythrocytosis, but is not sufficient for the phenotype to develop**. **JAK2 R1063H, with a weak effect on JAK2/STAT5 signaling is necessary to augment JAK2 activity caused by E846D above a threshold level leading to erythrocytosis and megakaryocytic atypia**. The presence of both mutations in the germline of rare PV and certain leukemia patients suggests that **they might be predisposing with respect to the development of hematological malignancy**. This study was published in ***Blood*** (Attachment 11).

2.3.3 *RUNX1* and *NF-E2* expression in polycythemia (Attachment 12)

In collaboration with prof. J.T. Prchal we aimed to analyze whether exaggerated erythropoiesis in various polycythemia states due to augmented EPO sensitivity is generally caused by increased transcripts of *RUNX1* and *NF-E2* as it was proposed for PV (Bogeska et al, 2013; Wang et al, 2010).

The study included twenty six subjects. Eighteen patients had mutation in JAK2, EPOR, VHL, HIF2A, or LNK and their erythroid progenitors exert *in vitro* hypersensitivity to EPO. Three of them were from the Czech Republic; two with G537R substitution in HIF2A and one with *EPOR* 5967insT mutation. Eight subjects with normal (non-hypersensitive) growth of erythroid progenitors *in vitro* were classified as secondary polycythemia. We found that **RUNX1 and NF-E2 overexpression is not specific for PV**. **Expression of RUNX1** and **NF-E2 was significantly elevated also in polycythemias with augmented HIF activity and EPO-hypersensitive erythroid progenitors**. On the other hand **RUNX1 and NF-E2 overexpression was not detected in patients with EPOR gain-of-function mutations** nor in secondary polycythemia subjects.

Our results suggest that **distinct mechanisms lead to EPO hypersensitivity of erythroid progenitors in polycythemias with defects of hypoxia sensing and those associated with EPOR mutations**. This study was published in ***Blood*** (Attachment 12).

2.3.4 Perinatal polycythemia correction in mice with human gain-of-function EPOR (Attachment 13)

Gain-of-function mutations of EPOR resulting from the truncation of the cytoplasmic domain of EPOR are associated with PFCP. A detailed analysis of a PFCP mouse model in which the murine EpoR gene (m*EpoR*) was replaced by either wild-type human EPOR (wtH*EPOR*) or mutant human EPOR (mtH*EPOR*) genes revealed that mtH*EPOR* mice become polycythemic at 3-6 weeks of age, but not at birth (Divoky et al., 2001). On the other hand, wtH*EPOR* mice are anemic (Divoky et al., 2001). In the presented study we observed that also mtH*EPOR* fetuses are polycythemic at the mid- and late-gestation stages (embryonic days, ED12.5-18.5), while wtH*EPOR* fetuses are anemic (ED12.5-16.5). The fetal polycythemia of mtH*EPOR* mice was associated with a delayed switch from primitive to definitive erythropoiesis and augmented and sustained activation of Stat5.

My direct contribution to this paper was the assessment of possible causes of inexplicable perinatal absence of polycythemia in mtH*EPOR* mice. We hypothesized that it may be related to the changes of oxygen tension at delivery from the hypoxic uterus to the normoxic ambient atmosphere and to differences in Epo levels.

At postnatal day 7 (PN7), mtH*EPOR* newborns experience the greatest decrease in hematocrit level (drop of 56%) when compared to wtH*EPOR* (drop of 24%) and m*EpoR* (drop of 48%) neonates. Neonatal mtH*EPOR* mice (PN7) had the lowest serum Epo levels, while wtH*EPOR* neonates had the highest Epo levels (Fig. 1). This may suggest a protective effect of Epo on the destruction of young (hypoxia-made) erythrocytes resulting in the non-polycythemic period in the mtH*EPOR* newborns.

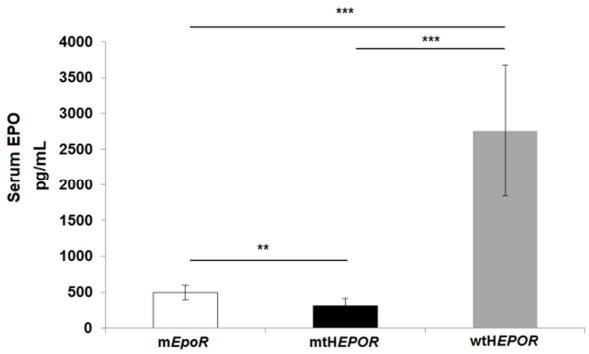
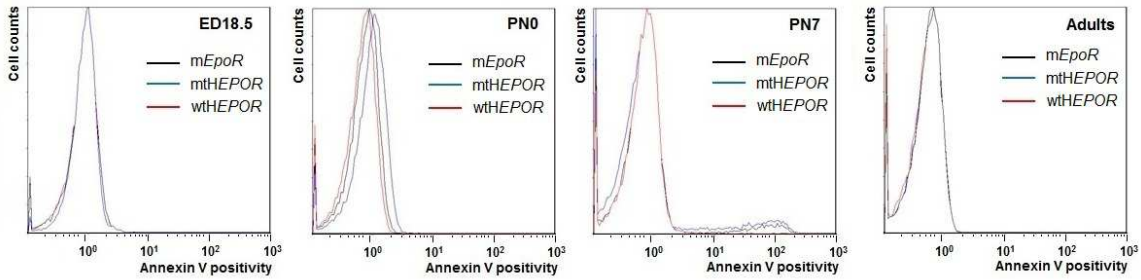


Figure 1. Neonatal (PN7) mtH*EPOR* mice have lowest Epo levels, while wtH*EPOR* mice have the highest Epo levels. ***P<0.001, **P<0.01, *P<0.05

In agreement we detected a gradual increase in the exposure of phosphatidylserines on erythrocytes membrane in mtH*EPOR* mice and to a lower extent also in m*EpoR* mice between postnatal day 0 (PN0) and PN7 with a maximum positivity at PN7 (Fig. 2). This corresponded to a maximum decrease of hematocrit and low Epo levels at PN7 in mtH*EPOR*. The exposure of phosphatidylserines then declines with the age to comparable levels in adult mice of all genotypes (Fig. 2). On the other hand wtH*EPOR* mice showed the lowest levels of phosphatidylserine exposure on erythrocytes with subtle changes in the perinatal period (Fig. 2).

A



B

Mean Fluorecsence (Annexin V)

	m*EpoR*	mtH*EPOR*	wtH*EPOR*
ED18.5	0.89 ± 0.17	0.91 ± 0.03	0.82 ± 0.05
PN0	0.93 ± 0.18	1.03 ±0.05	0.74 ± 0.07
PN7	6.17 ± 1.66	6.47 ± 2.77	1.33 ± 0.27
Adults	0.57 ±0.01	0.57 ± 0.05	0.57 ±0.04

Figure 2. mtH*EPOR* erythrocytes show increased phosphatidylserine exposure on erythrocytes membrane. Annexin V binding to exposed phosphatidylserines on the membrane of erythrocytes isolated from embryos at day 18.5 (ED18.5), neonatal mice at postnatal day 0 (PN0) and 7 (PN7), and adult mice (8-12 weeks old) was determined by flow cytometry. The overlays of original histograms of representative measurements are shown (top).

This is consistent with the lowest reduction in hematocrit and very high Epo levels in wtH*EPOR*. Increased phosphatidylserine exposure on the membrane of mtH*EPOR* erythrocytes is congruent with accelerated destruction of these cells by macrophages, thus contributing to the fall of hematocrit in neonatal mice.

We conclude that the **transient perinatal correction of polycythemia in mtH*EPOR* mice is associated with low Epo levels and increased exposure of**

phosphatidylserines on erythrocytes membrane. This study was published in J ***Mol Med (Berl)*** (Attachment 13).

3. Summary

Thanks to a conscientious effort initiated at the Department of Hemato-Oncology by prof. Karel Indrak and continued by Dr. Vladimir Divoky and prof. Dagmar Pospisilova at the Departments of Biology and Pediatrics, Faculty of Medicine and Dentistry, at Palacky University and University Hospital Olomouc, our departments have become a respected center for studies of congenital defects of erythropoiesis and iron metabolism. The close collaboration between the clinical practice and experimental research enabled us to unravel the molecular bases of congenital defects of erythropoiesis and iron metabolism in a substantial number of patients including the identification of novel disease causing variants.

As a part of my habilitation thesis we have revealed **multiple consequences of DMT1 deficiency on erythropoiesis** and clearly showed that **impaired function of DMT1 iron transporter negatively affects all stages in the erythroid lineage** and leads to severe hypochromic microcytic anemia. The defect can be, in part, corrected with high doses of EPO.

We also confirmed **disrupted erythropoiesis-hepcidin-iron stores axis in the selected types of congenital anemia:** PK deficiency, DBA, erythrocyte membrane defects, and thalassemia traits. The degree of deregulation reflected the severity of the disease. Our data supported the existence of another (or eventually other) erythroid-derived suppressor(s) of hepcidin. Very promising, among the potential molecules, seems to be the ERFE (Kautz et al., 2014). The human assay for ERFE measurement, which would enable to determine its levels under different pathological conditions and assess its correlation with hepcidin, is currently under development by Intrinsic LifeSciences (USA).

In addition, we showed that **hepcidin measurements may assist in differential diagnosis of different types of anemia**. **The combination of hepcidin levels with other parameters of iron metabolism and erythropoiesis, especially ferritin and sTfR is a powerful tool for assessing disease severity.** The understanding of the interactions and co-regulation of erythropoiesis and iron metabolism in the combination with the detailed characterization of molecular pathophysiology of disorders is an important step towards the development of novel therapeutic approaches. Multiple agents targeting hepcidin production or action are under development. Disorders with inappropriately low hepcidin, such as hereditary hemochromatosis and iron-loading anemia, might benefit from

treatment with agents, which potentiate hepcidin production (BMP pathway agonists) or mimic hepcidin action (eg. minihepcidins). On the other hand, agent that would reduce hepcidin activity or levels (hepcidin antagonists, eg. BMP pathway inhibitors or hepcidin peptide neutralizing binders), could be beneficial in iron-restricted anemias, especially anemia of inflammation or eventually anemia of cancer. Some of these drugs have already been successfully tested and the clinical trials are ongoing (reviewed in Ruchala and Nemeth, 2014).

Finally, we gain insights into the hypersensitivity of erythroid progenitors to EPO in different types of polycythemia and into the perinatal correction of polycythemia in the mtH*EPOR* mouse model of PFCP. The most important contribution is the **extension of the list of molecular lesions responsible for congenital erythrocytosis.** For the first time in the literature we clarified that the **cooperation of two weakly activating JAK2 germline mutations leads to the development of hereditary erythrocytosis with megakaryocytic atypia due to augmented EPO-dependent JAK2/STAT5 signaling**.

Collectively, I believe that the data presented in my habilitation thesis furthered our knowledge on the process of erythropoiesis and iron metabolism.

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5. Abbreviations

2,3 BPG – 2,3-bisphosphoglycerate
ADP – adenosine diphosphate
AGM – aorta-gonad-mesonefros
ALAS2 – 5'-aminolevulinate synthase 2
ATP – adenosine triphosphate
b/b – Belgrade rats
BFU-E – burst-forming unit-erytroid
BMP – bone morphogenic protein
BMPR – bone morphogenic protein receptor
Ca^{2+} – calcium ion
CDA - congenital dyserythropoietic anemia
CFU-E – colony forming unit-erythroid
CFU-GEMM (CFU-mix) – CFU granulocytic, erythroid, megakaryocyte, macrophage
CIS – cytokine-inducible SH2 protein
CP – ceruloplasmin
DBA – Diamond-Blackfan anemia
DCYTB – duodenal ferrireductases
DMT1 – divalent metal transporter 1
EB – erythroblast
ED – embryonic day
eIF2α – alpha subunit of the eukaryotic initiation factor – 2
EKLF – erythroid Krüppel-like factor
EPO – erythropoetin
EPOR – erythropoetin receptor
ERFE – erythroferrone
Fe – iron
Fe^{2+} – ferrous iron
Fe^{3+} – ferric iron
Fe-S – iron-sulfur clusters
Flt-1 – vascular endothelial growth factor receptor-1
FLVCR1 (Flvcr1) - feline leukemia virus, type C, receptor 1
FLVCR1a (Flvcr1a) – isoform 1a of FLVCR
FLVCR1b (Flvcr1b) – isoform 1b of FLVCR
FOG-1 – friend of GATA-1
FPN – ferroportin
G6PD – glucose-6-phosphate dehydrogenase
GDF15 – growth differentiation factor 15
GM-CSF – granulocytic, macrophage colony stimulating factor
GTP – guanosine-5'-triphosphate
Hb – hemoglobin
HE – hereditary elliptocytosis
HFE – protein associated with hemochromatosis type I
HH – hereditary hemochromatosis
HIF – hypoxia inducible transcription factor
HIFA (HIFα) – alpha subunit of HIF
HIFβ (ARNT) – beta subunit of HIF
HJV – hemojuvelin
HK – hexokinase
HO-1 – heme oxygenase-1
HO-2 – heme oxygenase-2
HRE – hypoxia response elements
HRI – heme regulated eIF-2α inhibitory kinase
HS – hereditary spherocytosis
HS40 – hypersensitivity site 40
HSC – hematopoietic stem cell
IDA – iron deficiency anemia

IGF-1 – insulin growth factor 1
IL-3 – interleukin-3
IL-6 – interleukin-6
IL-6R – IL6 receptor
INFγ – interferon γ
IRE – iron responsive element
IRIDA – iron refractory iron deficiency anemia
IRP – iron regulatory protein
JAK2 – Janus kinase 2
LCR – locus control region
LNK – lymphocyte adaptor protein
LPI – labile plasma iron
MapK – mitogen activated protein kinase
MARE – Maf - recognition element
mHJV – membrane-bound HJV
mk – microcytic anemia mice
MNC – mononuclear cells
MPN – myeloproliferative neoplasm
NF-E2 – nuclear factor erythroid-2
NRAMP1 – natural resistance associated macrophage protein 1
Nrf – NF-E2-related factor
NTBI – non-transferrin-bound iron
O_2 – oxygen
PFCP – primary familial and congenital polycythemia
PHD – prolyl-hydroxylase
PI3K – phosphatidylinositol-3 kinase
PK – pyruvate kinase
PN – postnatal day
PPIX – protoporphyrin IX
PV – polycythemia vera
VHL – von Hippel-Lindau
ROS – reactive oxygen species
RP – ribosomal protein
SCF – stem cell factor
SCL/TAL-1 – stem cell leukemia/T-cell acute leukemia
SH2 – src homology domain 2
sHJV – soluble HJV
SHP-1 – Src homology region 2 domain-containing phosphatase-1
SLC2A1 - solute carrier family 2 member 1 (glucose transporter-1)
SOCS – suppressor of cytokine signaling
STAT-3 – signal transducer and activator of transcription-3
STAT-5 – signal transducer and activator of transcription-5
STEAP 3 – six-transmembrane epithelial antigen of the prostate 3
sTfR – soluble transferrin receptor
Tf – transferrin
TfR (TfR1) – transferrin receptor-1
TfR2 – transferrin receptor-2
TGF-β – transforming growth factor-β
TM – thalassemia major
TMPRSS6 – type II transmembrane serine protease 6
TNF-α – tumor necrosis factor-α
TPO – thrombopoietin
TSAT – transferrin saturation
TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling
TWSG1 – twisted gastrulation
UTRs – untranslated regions
VEGF – vascular endothelial growth factor
wt – wild-type

6. Attachments

1. **Horvathova M**, Kapralova K, Zidova Z, Dolezal D, Pospisilova D, Divoky V. Erythropoietin-driven signaling ameliorates the survival defect of DMT1-mutant erythroid progenitors and erythroblasts. ***Haematologica***. 2012; 97:1480-8.

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Attachment 4

Mojzikova R, Koralkova P, Holub D, Zidova Z, Pospisilova D, Cermak J, Striezencova Laluhova Z, Indrak K, Sukova M, Partschova M, Kucerova J, **Horvathova M**, Divoky V.

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