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DISSERTATION

Titel der Dissertation

The regulation of iron metabolism in red blood cells and the direct effect of oxygen on erythropoiesis

Verfasser

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1. Zusammenfassung

Die Elemente Eisen und Sauerstoff sind für die Erythropoiese von besonderer Bedeutung. Eisen ist der essentielle Bestandteil von Häm, welches in Erythroblasten gebildet wird, um Sauerstoff zu binden und über den Blutkreislauf im ganzen Körper zu verteilen. Außerdem beeinflusst die Verfügbarkeit von Sauerstoff die Expression des Hormons Erythropoietin, dessen Konzentration im Körper die Neubildung von Erythrozyten reguliert. In dieser Arbeit wird mit Hilfe eines primären erythroiden Zellsystems gezeigt, wie es differenzierenden Erythroblasten gelingt, Eisenimport und Speicherung sowie die Synthese von Protoporphyrin IX so zu regulieren, dass eine optimale Abstimmung zwischen den einzelnen Mechanismen gewährleistet wird. Das ist deshalb von Bedeutung, da ein Überschuss an Eisen als auch an Protoporphyrin IX aufgrund ihrer Toxizität eine normale Erythropoiese behindern würde. Wir konnten zeigen, dass die Hauptbestandteile des zellulären Eisenstoffwechsels nicht so exprimiert werden, wie es das zelluläre Eisen Regulationssystem erwarten lässt. Die Ursache hierfür liegt in der Kopplung des Eisenimports mit der sofortigen Verwertung in den Mitochondrien, die trotz der gesteigerten Eisenaufnahme dazu führt, dass im Zytosol praktisch kein freies Eisen vorhanden ist. Zusätzliche Daten, die mit iron regulatory protein (IRP) defizienten Erythroblasten gewonnen wurden, zeigen, dass die beiden IRPs unterschiedlich relevant für die Regulation des Eisenstoffwechsels sind, was wiederum eine der Situation angepasste Expression der beteiligten Proteine erlaubt. Weiters konnten wir feststellen, dass sich eine Verknappung der Sauerstoffversorgung auf die Aktivität der IRPs auswirkt. Überraschenderweise ist dieser Effekt an die unter diesen Bedingungen geringere Bereitschaft der Erythroblasten vollständig zu differenzieren gekoppelt und wird deshalb nur indirekt durch die geringere Sauerstoffkonzentration verursacht. Vielmehr konnten wir zeigen, dass eine niedrige Sauerstoffkonzentration die Proliferation unreifer Erythroblasten begünstigt, während eine Erhöhung der Konzentration die vollständige Differenzierung ermöglicht. Diese Beobachtung korreliert mit den *in vivo* Gegebenheiten in hämatopoietischen Geweben. Hier befinden sich unreife Vorläuferzellen in Regionen mit geringer Sauerstoffversorgung, während differenzierende Zellen sich mit zunehmendem Reifegrad in Richtung höherer Sauerstoffkonzentration orientieren. Veränderungen der Sauerstoffversorgung in hämatopoietischen Geweben bewirken daher eine direkte Regulierung der Erythropoiese, zusätzlich zur indirekten Regulation über Erythropoietin. Ferner führt die Zugabe des HIF-Aktivators Dimethyloxalylglycin bei kultivierten Erythroblasten zu den gleichen Ergebnissen wie eine Inkubation bei niedriger

Sauerstoffversorgung. Daraus schließen wir, dass die Aktivierung von HIFs für die direkte, sauerstoffabhängige Regulierung der Erythropoiese verantwortlich ist.

2. Abstract

The chemical elements iron and oxygen are of special importance for the generation of red blood cells. Iron is essential for the formation of heme that is synthesized in erythroblasts in order to bind oxygen in the lungs and to distribute it all over the body via the blood circulation. Furthermore, oxygen influences the expression of the glycoprotein hormone erythropoietin, whose concentration regulates the production of erythrocytes. By using a primary erythroid cell culture system, we show how differentiating erythroblasts are able to regulate iron-import, iron-storage and synthesis of protoporphyrin IX in a way that allows an optimal balance between these three processes. This is of special importance since an excess of iron or protoporphyrin IX would interfere with normal erythropoiesis due to their toxic properties. We could show that the main components of cellular iron metabolism are not expressed as the conventional iron regulatory system implies. This is caused by the coupling of iron import with its instant processing in the mitochondria, which leads to a virtually iron free cytosol despite increased iron acquisition during erythroid differentiation. Additional data generated with iron regulatory protein (IRP) deficient erythroblasts show that the two IRPs are of different relevance for the regulation of iron metabolism in the erythroid system, which allows a flexible and situation-dependent expression of the relevant proteins needed for heme synthesis during terminal erythroid differentiation. In addition to iron, oxygen has been shown to influence the activity of IRPs as well. This is particularly important with cell culture systems. In fact we could show that a reduction of oxygen supply affected the IRP activity in differentiating erythroblasts. Surprisingly, this effect was coupled to a reduced ability of the cells to differentiate properly and therefore was only indirectly caused by the low oxygen levels. Actually, we could show that low oxygen levels promote the proliferation of immature erythroblasts while an increase of the oxygen concentration allows terminal differentiation. This observation correlates with the *in vivo* situation in hematopoietic tissues. Immature progenitor cells reside in regions that are hypoxic whereas differentiating cells migrate to increasingly oxygenated areas the more mature they become. Consequently, changes of the oxygen supply of hematopoietic tissues allow the modulation of erythropoiesis directly, additionally to the indirect induction via erythropoietin. Furthermore, addition of the HIF-activator dimethyloxalylglycine resulted in the same effects observed with incubation at low oxygen levels. Therefore, we conclude that the activation of HIFs during systemic hypoxia is sufficient for a direct oxygen dependent regulation of erythropoiesis.

3. Introduction

3.1. Hematopoiesis

Hematopoiesis comprises the formation of leukocytes, erythrocytes and thrombocytes. These cells are required for the immune response, the oxygen transport and the blood coagulation in case of injury. Since blood cells in general have a limited life span, the organism is forced to continuously replenish lost cells. This is achieved by the self renewal and differentiating capacity of the multipotent long-term hematopoietic stem cells (LT-HSC)[1]. The self renewal capacity guarantees the survival of LT-HSCs in its niche as a source for all types of blood cells[2]. Differentiation leads to the formation of more committed cell types, starting with short-term hematopoietic stem cells that further differentiate into different types of multipotent progenitors (MPP) including lymphoid primed multipotent progenitor, the common myeloid or the common lymphoid progenitor (Figure 1), although the exact sequence and branching points of lineage commitment are still under discussion[3]. The MPPs further differentiate into different lineages and committed precursors and finally develop into fully differentiated mature blood cells. In general, the self renewal capacity of the hematopoietic cells decreases with each differentiation step. Furthermore, the entire process of hematopoiesis is dependent on the activity of several transcription factors that cooperate to regulate the decision between self renewal and differentiation[1,4]. The environment of the hematopoietic cells therefore plays a major role in their maintenance and differentiation by regulating the activities of these transcription factors[3,5]. This is also reflected in the change of the location of the hematopoietic tissue during development. The first organ of hematopoiesis in the murine embryo is the yolk sac, followed by the aorta-gonad mesonephros (AGM) region and the chorio-allantoic placenta[6]. Subsequently, hematopoiesis takes place in the fetal liver, followed by the bone marrow after birth. Each of these organs favors the production of specific blood cells. For instance, during the fetal liver phase the main part of hematopoietic capacity is focused on the production of erythrocytes in order to supply the growing embryo with sufficient amounts of oxygen.

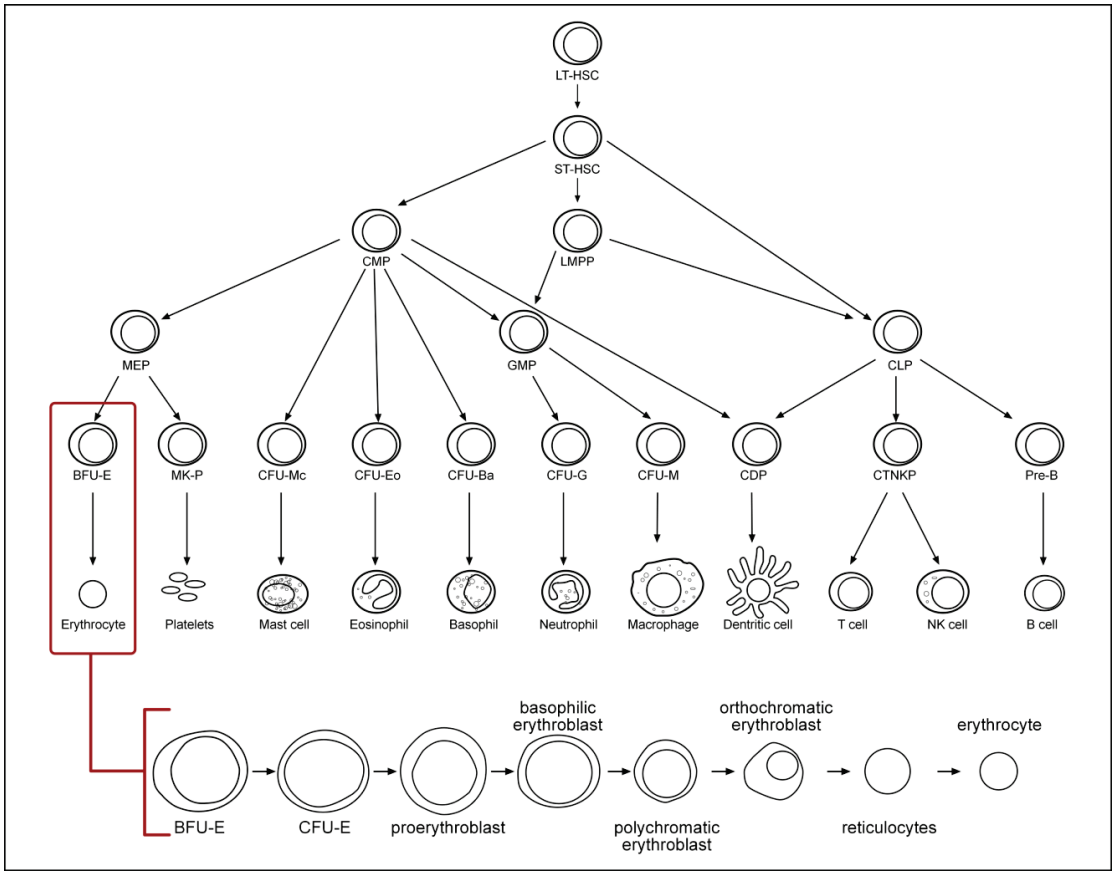

Figure 1. Schematic overview of hematopoiesis with a detailed look at erythropoiesis. The long term hematopoietic stem cell (LT-HSC) is able to self renew or to differentiate in more mature hematopoietic precursor cells giving rise to all the hematopoietic lineages. As indicated by the arrows, the lineage commitment of early progenitors is not totally restricted but includes a certain degree of plasticity. For simplicity, the final steps of lineage differentiation are not shown in detail and involve several additional cell stages. The lower panel shows erythropoiesis in more detail, starting with the BFU-E and finally resulting in the formation of the mature erythrocyte.

Figure adapted and simplified from Miranda-Saavedra and Göttgens, Current opinions in genetic and development65 2008[4] and A.Rad, Wikimedia.commons, 2011 http://commons.wikimedia.org/wiki/File:Hematopoiesis_%28human%29_diagram.png

3.2. Erythropoiesis

3.2.1. Ontogeny of erythropoiesis

Erythropoiesis is a special branch of hematopoiesis and is responsible for the generation of red blood cells. Primitive erythropoiesis begins at embryonic day 7.5 (E7.5) in the yolk sack and guarantees the oxygen supply of the early developing embryo[7]. The first primitive erythroid cells enter the circulation around E8.5 with the onset of the heartbeat. Their maturation is still in progress after the release from the yolk sac into the circulation and comprises hemoglobin accumulation, globin type switching and enucleation[8-10]. At E9.5 yolk sac derived progenitor cells initiate the definitive erythropoiesis in the fetal liver[11]. By E11.5 they are displaced by HSCs of the AGM-region that continue with the production of red blood cells until erythropoiesis is transferred to the bone marrow after birth[6,12].

Definitive erythropoiesis in mammalians results in the production of erythrocytes that are characterized by a biconcave shape and the lack of the nucleus, mitochondria and other organelles[13]. Furthermore, they are packed with hemoglobin in order to carry out their function as oxygen transporters. The major part of the maturation process of definitive erythroblasts takes place in the hematopoietic organs and only enucleated reticulocytes are released into the blood stream to become mature erythrocytes[14]. The specialized anatomical unit of erythropoiesis within the fetal liver and the bone marrow is the so called erythroblast island[15]. It consists of one or two central macrophages surrounded by several erythroblasts. During maturation the erythroblasts migrate from the center of the island to its borders and finally are released into a blood sinusoid. During the last steps of this process the cell's nucleus is released and endocytosed by the central macrophage.

3.2.2. Erythroid development

Erythropoiesis is part of the myeloid lineage of hematopoiesis. Therefore, the progenitors of erythroid cells are HSCs that differentiate into the common myeloid progenitor and subsequently into the megakaryocyte/erythroid progenitor. Further differentiation results in the blast forming unit erythrocyte (BFU-E; Figure 1), the first committed precursor that is restricted to the erythroid lineage. The BFU-E is dependent on the presence of stem cell factor (SCF) and erythropoietin (epo) and has a relatively high proliferation capacity: one single BFU-E can give rise to several thousand erythrocytes[16,17]. The BFU-E is

succeeded by the colony forming unit erythrocyte (CFU-E), a more committed progenitor that is dependent on the availability of epo and insulin or insulin-like growth factor 1[18-20]. Further differentiation leads to the proerythroblast that is limited to 3-4 additional cell divisions, which result in the sequential formation of basophilic, polychromatic and orthochromatic erythroblasts[21]. The transition from the proerythroblast to the orthochromatic erythroblast includes cell size decrease, condensation of the nucleus, increased production of hemoglobin and changes in the membrane organization[22,23]. Finally, orthochromatic erythroblasts release the condensed nucleus and become reticulocytes that detach from the erythroblastic island and enter the circulation to become mature erythrocytes.

3.2.3. Regulation of steady state and stress erythropoiesis

The normal turnover of erythrocytes makes it necessary to constantly produce new red blood cells. Nevertheless, in special situations like embryonic development or during insufficient oxygen supply due to blood loss or caused by a transfer to elevated altitudes the demand for red blood cells becomes higher than provided by steady state erythropoiesis. In this situation the body reacts by increasing the production rate of erythrocytes, a process called stress erythropoiesis. This response is tightly regulated by the interplay of signals transmitted by the SCF receptor (c-Kit), the epo receptor (epoR) and the glucocorticoid receptor (GR)[24,25].

The epoR and epo are essential for definitive steady state as well as stress erythropoiesis and their deletion leads to death at E12.5[26,27]. Upon binding of epo to the epoR, the epoR-associated Janus kinase 2 (Jak2) activates several signal cascades involved in the maturation of red blood cells[28,29]. This includes activation of the signal transducer and activator of transcription 5 (Stat5). The Bcl-2 gene family members Bcl-X_L and Mcl-1 belong to the targets of Stat5 and prevent apoptosis of differentiating erythroblasts if activated by epo signaling[30-32].

SCF and its receptor c-Kit are essential for the development of several hematopoietic lineages and promote proliferation of hematopoietic progenitors. The absence of functional SCF or c-Kit causes severe anemia and premature death[33]. Regarding erythropoiesis, c-Kit mediated signaling stimulates expansion and delays differentiation of hematopoietic/erythroid progenitors including the BFU-E[34]. The SCF/c-Kit complex activates the PI3K as well as the Ras/MAPK/ERK pathway. These signals are further enhanced by the formation of a joined signaling complex with the epoR, which

also activates these pathways, although less intensely[26,35-37]. Furthermore it has been shown that the Jak2 dependent activation of the epoR-target Stat5 is induced by c-Kit as well[38].

Glucocorticoids in combination with SCF and epo increase the proliferation capacity of erythroid cells and interfere with terminal erythroid differentiation[39,40]. Mice with a mutation of the GR that prevents binding of the transcription factor to its target sequence are viable and show normal steady state erythropoiesis[41]. However, these mice are not able to induce stress erythropoiesis during reduced oxygen supply indicating the importance of GR signaling under hypoxic conditions[42].

Taken together, the combination of SCF, epo and glucocorticoids induces stress erythropoiesis and can be used *in vitro* to establish cultures of proliferating primary erythroblasts that resemble BFU-Es and CFU-Es. These cells can be induced to undergo synchronous terminal differentiation by withdrawing SCF and replacing glucocorticoids by glucocorticoid-antagonists[43]. The close resemblance of this system to *in vivo* erythropoiesis makes it a perfect tool to analyze erythropoiesis as well as special aspects that are linked to this process.

3.3. Iron metabolism

Iron is essential for almost all cells due to its ability to easily transfer electrons in chemical reactions and therefore mediating catalytic reactions as well as due to its flexibility in the association with proteins and oxygen[44]. Hence, iron plays a crucial role in energy metabolism and DNA synthesis and is well known for its function in oxygen transport by hemoglobin in vertebrates. Nevertheless, the same abilities are also responsible for toxic effects, namely the generation of reactive oxygen species and other radicals, reactions that are summarized under the term Fenton chemistry[45]. Additionally, the oxidized trivalent form of iron (Fe^{3+}) shows limited solubility in aqueous solutions and has to be sequestered by specific proteins in order to prevent accumulation of precipitates of iron in tissues and plasma. Therefore, iron uptake, utilization and storage have to be tightly regulated in order to prevent the occurrence of oxidative stress that would lead to damage of cellular macromolecules and tissue injury[46].

3.3.1. Systemic Iron metabolism

The major part of body iron can be found in erythrocytes and developing erythroblasts. More than 2.1 g circulate in the blood of an average adult human incorporated in hemoglobin[47]. Macrophages that are responsible for recycling of iron derived from senescent erythrocytes contain 0.6 g iron. Further 0.3 g can be found in muscle cells embedded in myoglobin. Excess iron is stored in the liver, where up to 1 g can accumulate in hepatocytes under normal non-pathological conditions[48,49]. The remaining iron is distributed over the different tissues of the body. Surprisingly, only 2.5-3 mg of iron are actually directly available for the organism in the form of iron saturated plasma transferrin (Tf-Fe_2). This indicates the highly dynamic nature of the daily iron turnover, since 20-30 mg of iron, 10 times as much, are processed each day. As already indicated by the iron distribution in the body, the major part of the turnover is used for heme synthesis in developing erythroblasts (Figure 2). Under normal conditions, the body absorbs only 1-2 mg iron each day in the proximal small intestine since the iron needs are for the most part covered by the recycling and release of heme-bound iron of senescent erythrocytes. Iron absorption compensates for the iron loss caused by sloughing of intestine and skin cells as well as loss of iron associated with menstruation and the excretion of urine and bile.

The body can import iron in the form of heme as well as non-heme iron. Both are absorbed at the apical brush boarder of intestinal enterocytes (Figure 2). In the case of heme import the corresponding transporter is not known. The only candidate up to date is the solute carrier 46A1 (SLC46A1) but its primary function seems to be folate and not heme transport[50]. After heme import, iron is separated from protoporphyrin by heme oxygenase 1 (HOX1) and enters the cytosolic iron pool of the enterocyte[51]. Non-heme iron import is performed by the divalent metal ion transporter 1 (DMT1, SLC11A2)[52] and is dependent on the ferrireductase activity of duodenal cytochrome B (DcytB, CYBRD1) that transforms Fe^{3+} into Fe^{2+} [53,54]. At the basolateral membrane of enterocytes ferroportin (SLC40A1) exports cytosolic Fe^{2+} into the circulation[55,56]. The export is linked to the oxidization of Fe^{2+} to Fe^{3+} by hephaestin that additionally allows the binding of iron to the plasma protein transferrin (Tf)[57,58].

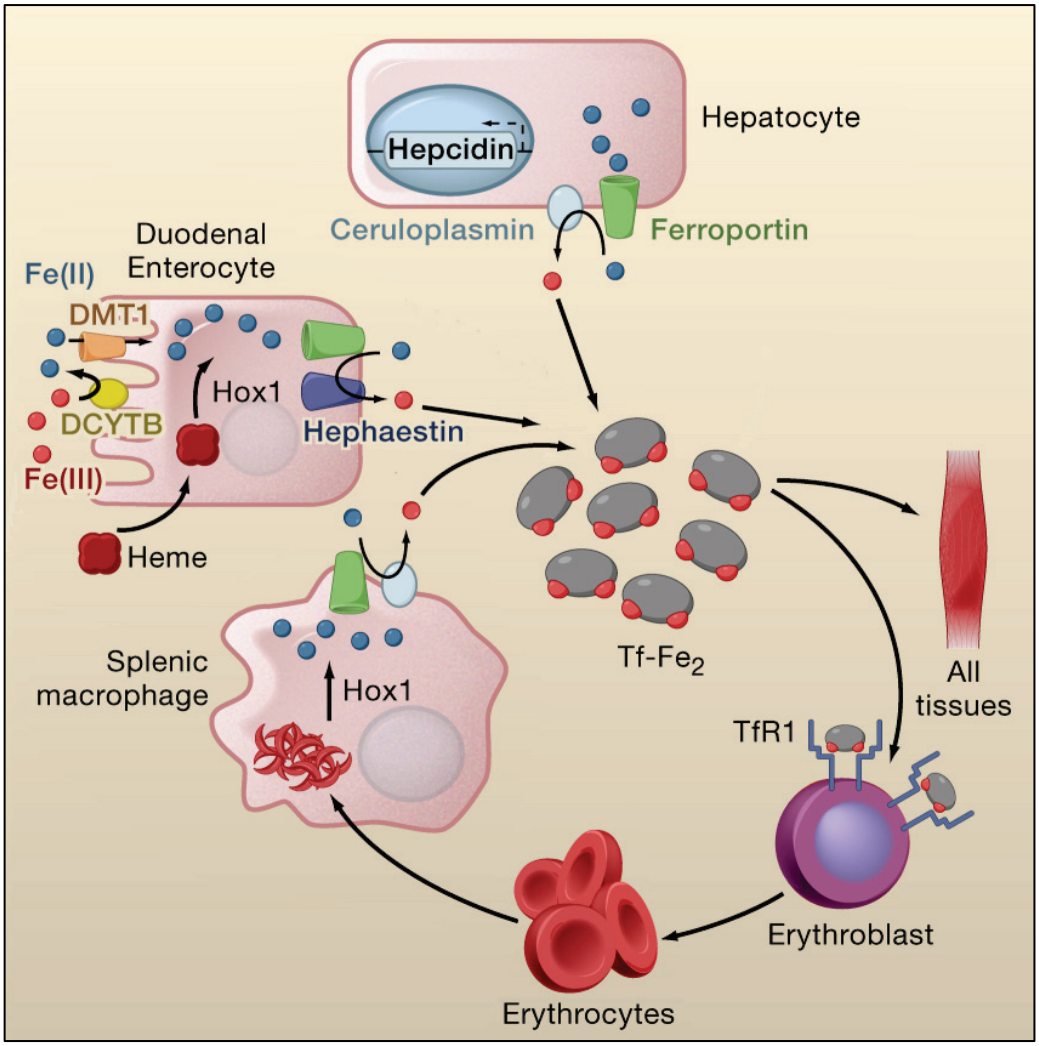

Figure 2. Systemic iron metabolism. Dietary iron is imported by duodenal enterocytes and released into the plasma were it binds to transferrin (Tf). The major part of the circulating iron is used by differentiating erythroblasts in order to synthesize heme whereas the iron consumption by all other tissues contributes less to the overall iron turnover. Senescent erythrocytes are recycled by splenic macrophages and their iron is released into the plasma, making the major contribution to the daily iron turnover while the duodenal iron import compensates for the systemic loss of iron caused by sloughing and bleeding (not shown). The liver has an iron storage function and releases iron if the body is confronted with iron deficiency. Furthermore, hepatocytes express hepcidin that induces the degradation of ferroportin in the case of iron overload and therefore play an important role in the regulation of systemic iron metabolism. For detailed description of distinct proteins see text. Figure adapted from Hentze et al., Cell 2010[59].

3.3.2. Cellular iron uptake and trafficking

Tf is responsible for the iron supply of most of the cells within the body. The iron loaded form (Tf-Fe_2) has a relatively high affinity for the transferrin receptor (TfR1) compared to the iron depleted form (apo-Tf) and is therefore preferentially bound. The Tf-Fe_2/TfR1 complex formation triggers clathrin dependent endocytosis and subsequent acidification of the endosomal compartment by proton pumps leads to a conformational change that releases Fe^{3+} from Tf but keeps the Tf/TfR1 complex intact[60] (Figure 3). The ferrireductase Steap3 converts Fe^{3+} into Fe^{2+} and DMT1 transports iron across the endosomal membrane into the cytosol[61]. The Tf/TfR1 complex is redirected to the plasma membrane where the readjustment of the pH leads to the release of the iron depleted apo-Tf. While the TfR1 is now able to bind another Tf-Fe_2, apo-Tf can be reloaded with Fe^{3+} released by enterocytes, hepatocytes or macrophages (Figure 2).

Tf/TfR1 independent import is limited to special cell types and physiological situations and therefore plays a minor role in general iron metabolism. Erythropoiesis for example is strictly dependent on iron supply by TfR1 as its targeted disruption leads to early lethality of murine embryos due to severe anemia[62]. Confirming the importance of the Tf/TfR1 pathway, $TfR1^{+/-}$ mice are not able to provide developing erythroblasts with sufficient amounts of iron and develop microcytic hypochromic anemia. Furthermore, Tf deficient humans and mice develop severe iron deficiency anemia[63,64]. Nevertheless it is still under discussion whether macrophage derived ferritin makes a contribution to the iron supply of developing erythroblasts[65,66]. A well known alternative route of cellular iron uptake that is restricted to macrophages is the already mentioned phagocytic recycling of senescent erythrocytes (Figure 3). Macrophages are also able to directly internalize heme (bound to plasma hemopexin)[67] and hemoglobin (bound to haptoglobin)[68]. The iron supply through the blood brain barrier is not well understood but probably involves mechanisms comparable to the iron uptake in the duodenum[69,70]. Further alternative iron uptake pathways are connected to anti inflammatory reactions[71,72] and pathological states linked to iron overload[73] and extracellular ferritin[74,75].

The iron transport mechanisms within the cell are not well understood. When iron enters the cytosol it becomes part of the transient labile iron pool (LIP) that can be detected with the help of fluorescent sensors (reviewed in Breuer et al, 2008)[76]. The substances that bind iron of the LIP are still not defined but may include citrate, peptides, ATP or different phosphates. Nevertheless, the major part of cellular iron does not stay in the cytosol but is directed to mitochondria, where it is used for the synthesis of heme and iron sulfur clusters (Fe/S cluster; Figure 3). In yeast it has been shown that Grx3p and

Grx4p are essential for iron sensing in the cytosol and its transport to mitochondria[77]. It is still discussed whether differentiating erythroid cells bypass the cytosol by direct interaction of endosomes with mitochondria[78] or use the conventional iron transport pathway involving the LIP[79]. The iron transporters mitoferrin1 (Mfrn1) and Mfrn2 are responsible for the mitochondrial iron import. Mfrn1 is essential for effective erythroid differentiation[80] and is stabilized by the interaction with the erythroid specific ABC transporter Abcb10[81]. Mfrn2 is ubiquitously expressed but is not upregulated during increased mitochondrial iron demand and therefore cannot compensate for the loss of Mfrn1 that leads to severe defects in heme synthesis and Fe/S cluster assembly[82].

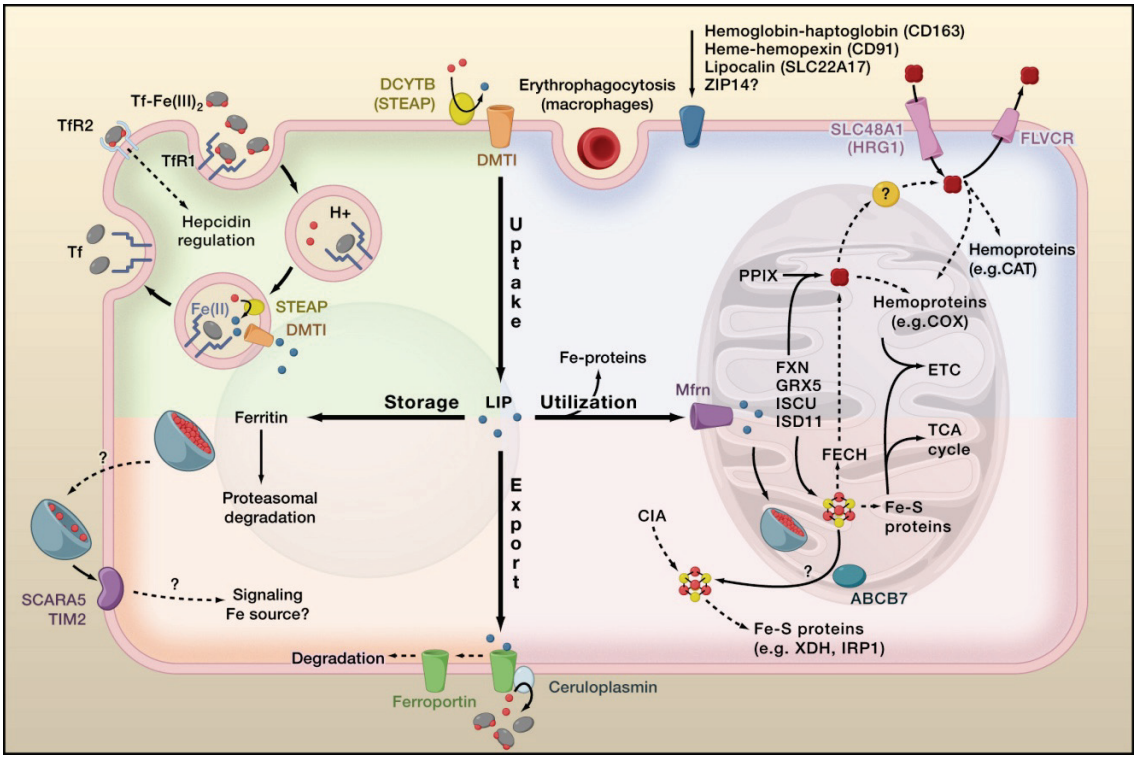

Figure 3. Cellular iron metabolism. Note that some of the components are only available and active in certain cell types, e.g. iron import in most cell types is dependent on the TfR1, while macrophages are able to phagocytose senescent erythrocytes and duodenal enterocytes transport dietary iron directly via DMT1. Furthermore, iron export of enterocytes is linked to the oxidative activity of hephaestin instead of ceruloplasmin. For further details see text. Figure from Hentze et al., Cell 2010[59].

3.3.3. Iron utilization – heme synthesis and Fe/S cluster assembly

Mitochondria are responsible for the synthesis of heme and Fe/S clusters and therefore are the main consumers of cellular iron. Heme is not only needed for the transport of oxygen but is also part of several hemoproteins that are involved in oxidative metabolism, thyroid hormone synthesis and other cellular processes. The protoporphyrin/heme synthesis pathway starts with the reaction of succinyl-CoA with glycine that leads to the formation of δ-amino levulinic acid (ALA; heme synthesis and transport reviewed in Severance et al. 2009)[83]. This reaction is catalyzed by either of the two homologue enzymes ALA synthase 1 (ALAS1) and ALAS2 (ALAS-E)[84-86]. ALAS1 is ubiquitously expressed and responsible for the baseline synthesis of heme whereas ALAS2 expression is limited to erythrocytes and allows increased heme synthesis during erythropoiesis[87,88]. Furthermore, ALAS2 expression is linked to iron availability and connects heme synthesis with iron supply (see below). ALA is exported into the cytosol where it is processed to coproporhyrinogen III involving four sequential enzymatic reactions. Subsequently, coproporhyrinogen III is imported into mitochondria and oxidized to protoporphyrin IX (PPIX). In the last step of the heme synthesis pathway, ferrochelatase catalyzes the insertion of Fe^{2+} into PPIX. Heme is then incorporated into heme dependent proteins in mitochondria, the cytosol, the endoplasmatic reticulum and the nucleus. Little is known of the mitochondrial export of heme or of the transport of heme to the places of hemoprotein assembly. Since free heme is hydrophobic and cytotoxic it is postulated that it involves specific molecules and pathways[89].

Fe/S clusters are part of several enzymes and proteins that are essential for electron transfer, energy metabolism, lipoate synthesis, nucleotide metabolism and iron metabolism[90]. The functions of the Fe/S clusters as part of these proteins include electron transfer, stabilization of protein structure and regulation of enzyme activity. The Fe/S cluster synthesis depends on a complex pathway involving several components and despite recent advances in the understanding of the cluster formation there still remain several open questions. There is evidence that the iron chaperone frataxin is responsible for the delivery of iron to the Fe/S cluster formation machinery although this issue is still controversial and the exact mechanism is not known[91,92]. Besides the Fe/S cluster assembly machinery in the mitochondria several homologue components of the machinery could also be detected in the cytosol and the nucleus. Although one model proposes totally independent de novo Fe/S cluster assembly machinery in the cytosol[93] there is evidence that cytosolic cluster assembly is dependent on the mitochondrial machinery[94-98]. Defects in the generation of Fe/S clusters result in deregulation of iron

metabolism and are linked to several diseases like Friedreich's ataxia or X-linked sideroblastic anemia with cerebellar ataxia[90,99].

3.3.4. Cellular iron export and storage

Ferroportin is the only known cellular exporter of elemental iron[55,56]. The function of the transporter is linked to ferroxidase activity of hephaestin in enterocytes and ceruloplasmin in all other cell types[100]. Disruption of ferroportin is embryonically lethal since the iron transfer via the extraembryonic visceral endoderm and the placenta is ferroportin dependent[101]. The targeted deletion of ferroportin that does not affect embryonic iron supply results in the accumulation of iron in macrophages, hepatocytes and duodenal enterocytes and leads to severe anemia, demonstrating the important role of ferroportin for systemic iron metabolism. Additionally to the cellular export of elemental iron, cells are able to export iron in the form of heme via FLVCR1[102]. The physiological function of this transporter is not well understood but it is linked to erythroid maturation and recycling of heme-iron by macrophages.

When the cellular iron demand is low, excess iron must be stored in a non toxic form within the cell. For this purpose the iron storage proteins ferritin H (FtH1) and ferritin L (FtL) assemble in a shell like structure containing 24 ferritin subunits[103] (Figure 3). FtH1 has an intrinsic ferroxidase activity while FtL contains a nucleation center that facilitates accumulation of excess iron. Up to 4500 Fe^{3+} ions can be stored in the form of ferric oxyhydroxide phosphate in a single ferritin cavity. Little is known about the transfer of iron from the LIP to the ferritin shell. It has been shown that Poly(rC)-binding protein 1 promotes iron loading of ferritin *in vitro* and is essential for iron loading in cell culture experiments[104]. The importance of iron storage is underlined by the early embryonic lethality of FtH1 knock out mice[105]. Conditional deletion of FtH1 leads to liver damage and disturbed iron absorption in the duodenum[106,107]. Mutations of FtL are linked to neurodegenerative disorders due to increased iron dependent redox activity in the brain[108]. Remobilization of ferritin bound iron involves lysosomal turnover[109] or is linked to ferroportin dependent export of iron that is followed by proteasomal degradation of iron depleted ferritin[110,111]. However, it has been shown that macrophages are not able to use ferritin bound iron as iron source for induced endogenous heme synthesis[112]. Ferritin can also be found in the serum and is used as a clinical marker of anemia since its levels correlate with tissue iron stores but are also increased during inflammation. It has been

shown that serum ferritin is excreted mainly by splenic macrophages and proximal tubule cells of the kidney[113].

An H-type ferritin isoform known as mitochondrial ferritin encoded by a nuclear intronless gene is targeted to mitochondria by an N-terminal leader sequence[114]. It is normally expressed at low levels but its expression is elevated in iron loaded erythroblasts (ring sideroblasts) during sideroblastic anemia[115] and therefore is thought to protect mitochondria from toxic effects of excess iron.

3.3.5. The two regulation systems of iron metabolism

Iron is essential for several physiological functions in the organism but at the same time excess iron harms cells and tissues. Since there is no mechanism that allows the active excretion of iron, regulation of dietary iron import is the only possible way to control its total amount in the body. The central regulator of systemic iron metabolism is the peptide-hormone hepcidin (reviewed by Nemeth and Ganz, 2009)[116]. Hepcidin is produced in the liver as an 84 amino acid propeptide that is cleaved into its 25 amino acid active form, which is then released into the blood[117]. It inhibits cellular iron export by binding to the iron transporter ferroportin. The complex formation triggers its own JAK2 dependent phosphorylation followed by internalization and lysosomal degradation[118,119]. Thus, if hepcidin levels are high, nutritional iron cannot enter the plasma since it is literally trapped within duodenal enterocytes that are removed through naturally occurring sloughing in the intestine within several days. Iron stores in hepatocytes and heme derived iron recycled by macrophages are retained by the same mechanism in order to reduce plasma iron levels[120,121]. Pathologically reduced levels of hepcidin result in iron overload or hemochromatosis and consequently lead to liver cirrhosis, cancer, diabetes, hypogonadism, heart failure and arthritis if not treated by iron depletion therapy[122]. Pathologically high levels of hepcidin result in iron deficiency anemia[123,124]. The expression of hepcidin is regulated transcriptionally and integrates signals of systemic iron supply[122,125,126], inflammation[127-129], oxidative stress[130] and erythropoiesis[131-133]. The pathways involved in the transduction of these signals include the BMP6/SMAD and the C/EBPα pathways as well as STAT3 and TLR4 signaling.

Additional to the hepcidin-dependent regulation of systemic iron metabolism, the posttranscriptional regulation of cellular iron uptake, storage and utilization is mediated by the iron regulatory protein 1 (IRP1; ACO1) and IRP2 (IREB2)[47,59]. The IRPs are able to bind to cis-acting hairpin structures of their target mRNAs, the iron responsive

elements (IRE). IREs are conserved in the majority of metazoa[134] and up to date have been found in the 3' untranslated region (UTR) of TfR1[135], DMT1[136], myotonic dystrophy kinase-related Cdc42-binding kinase α[137] and human Cdc14A[138] as well as in the 5' UTR of ferritin H and L[139], ferroportin[140], ALAS2[86], mammalian mitochondrial aconitase[141], drosophila succinate dehydrogenase[142], Alzheimer amyloid precursor protein[143], α-synuclein[144] and HIF2α[145]. IRP binding to 3' UTR IREs results in the stabilization of the corresponding mRNA whereas binding to the 5' UTR IREs interferes with translational initiation (Figure 4). The canonical IRE contains a conserved loop (CAGUGN) and a variable stem-sequence[146]. The α-helix formed by the stem is distorted by an unpaired C-bulge or a UGC/C-bulge/loop in the case of ferritin mRNA[147,148]. HIF2α and DMT1 mRNAs contain a non canonical IRE with an additional bulge in the upper stem. The C-bulge and the loop directly interact with the IRPs[149] but there is also evidence for variation of IRP1 binding affinity dependent on the stem sequence[150].

The IRPs belong to the family of iron-sulfur-cluster isomerases and are closely related to the mitochondrial aconitase, which is responsible for the conversion of citrate to isocitrate during the citric acid cycle[151]. The bifunctional IRP1 becomes the cytosolic isoform of the mitochondrial aconitase when it binds a 4Fe-4S cluster. Simultaneously, this inhibits the interaction with IREs, since the binding sites for the IRE and the Fe/S cluster overlap[149,152]. Therefore, the switch between IRE binding and aconitase activity is dependent on iron availability and a functional mitochondrial Fe/S cluster synthesis. IRP2 shares large homology with IRP1 but does not bind a Fe/S cluster and has no aconitase activity. In fact, the IRE binding activity of IRP2 is regulated by proteasomal degradation initiated by iron dependent ubiquitination. The F-box and leucine-rich repeat protein 5 (FBXL5), which is part of an E3 ubiquitin ligase complex, is responsible for the ubiquitination [153,154]. FBXL5 contains a hemerythrin domain with a Fe-O-Fe center making its function dependent on the availability of iron and oxygen. If iron levels are high, the enzyme is active and ubiquitination of IRP2 takes place. Upon iron depletion FBXL5 loses its functionality and becomes degraded.

In addition, IRPs are also responsive to non-iron signals. As already indicated above, hypoxia favors stabilization of IRP2 but also of Fe/S clusters, thereby decreasing IRP1 binding activity[155]. Reactive oxygen and nitrogen species activate IRP1 binding activity by destabilizing the bound Fe/S cluster[156] and there is evidence that nitric oxide as well as oxidative stress stabilize IRP2[157,158]. Furthermore, both IRPs are targets for specific phosphorylation influencing their binding activity and stability[159-162]. Taken together, these regulatory mechanisms allow the fine tuning of IRP binding activity additional to the regulation by iron availability.

The importance of IRP dependent regulation of cellular iron metabolism is demonstrated by IRP1/IRP2 double deficient mice that die during embryonic development at the blastocyst stage[163]. Targeted deletion of both IRPs in the duodenum results in intestinal mal-absorption and death within 4 weeks after birth[164]. In these mice iron import of enterocytes via TfR1 and DMT1 is decreased whereas iron storage by ferritin H and L as well as iron export by ferroportin are increased leading to cellular iron depletion and intestinal dysfunction. Mice with IRP deficient livers show mitochondrial iron deficiency and dysfunction of hepatocytes[165]. This leads to compromised heme and Fe/S cluster synthesis and premature death due to liver failure. IRP1 and IRP2 single knockout mice are both viable and fertile showing that their function is essential but partly redundant. IRP1 deficiency shows no pathological phenotype besides misregulation of ferritin and TfR1 expression in the kidney and brown fat tissue[166]. IRP2 knockout mice develop a mild microcytic anemia and show excessive iron deposits in the duodenum and the liver as well as iron deficiency in the spleen[167,168]. Furthermore, these mice also show a tendency for neurodegeneration linked to accumulation of iron in the brain[169]. The severity of this neuropathology is still under discussion and may be influenced by different target strategies[170,171]. Targeted deletion of IRP2 in enterocytes, hepatocytes or macrophages only affects the intrinsic iron metabolism of the respective cells and does not alter plasma iron levels indicating that microcytic anemia in IRP2 knockout mice is a cell autonomous effect as well[172].

Although the involved mechanisms of activation and action are different, the cellular regulators IRP1 and IRP2 and the systemic regulator hepcidin do not operate isolated from each other[59]. They both control the uptake of dietary iron as well as the release of iron into the plasma by directly regulating the abundance of ferroportin. The expression of hepcidin itself is modulated by the IRP-controlled iron availability in hepcidin-producing hepatocytes. Furthermore, the HIF transcription factors regulate the expression of hepcidin, ferroportin and DMT1[173,174] whereas HIF2α expression is controlled by IRP binding activity[145]. A fourth connection between the two regulatory systems is TfR1. The expression of the receptor is regulated by the IRE/IRP system but the receptor itself interacts with the HFE protein, which participates in the systemic iron status mediated control of hepcidin expression[175,176]. Since the regulation of hepcidin expression is still not fully understood and additional IRP targets are likely to be found, further connections may be revealed in the future. Nevertheless, the above mentioned mechanisms already illustrate the complexity and importance of proper iron regulation in each single cell as well as in the whole organism.

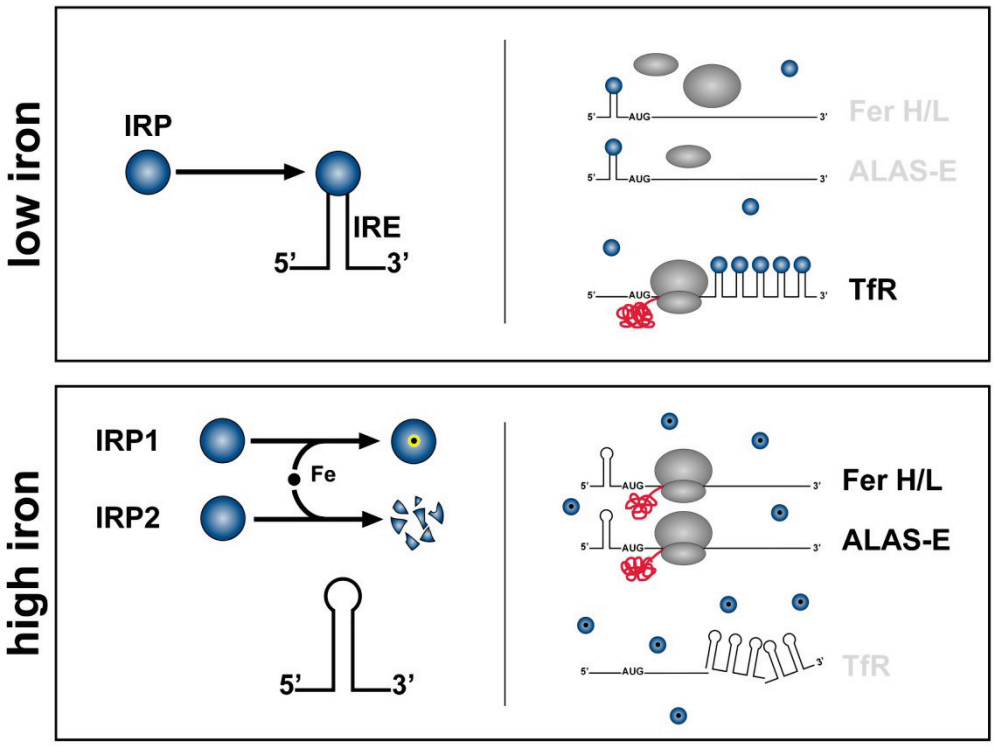

Figure 4. The IRE/IRP system – regulation of cellular iron metabolism. Iron deficiency increases the binding activity of IRPs and results in the downregulation of mRNAs containing a 5' IRE (e.g. ferritin or ALAS2) due to inhibition of translational initiation. 3' IRE-containing mRNAs (e.g. TfR1) are stabilized leading to increased expression of the corresponding proteins. If sufficient iron is available, the IRP1 binding activity is blocked by the incorporation of an iron sulfur cluster while IRP2 is degraded resulting in unbound IREs. This leads to the translation of 5' IRE-containing mRNAs while 3' IRE containing mRNAs become accessible to degradation. See text for further details.

3.4. Oxygen and the organism

Oxygen is one of the most abundant elements in our environment. It does not only contribute 21% to the earth's atmosphere but also can be found in large amounts in the lithosphere, the oceans as well as the biosphere of our planet. Furthermore, the circulation of oxygen between the biosphere and the atmosphere is very dynamic due to the oxygenic photosynthesis performed by cyanobacteria, green algae and terrestrial plants. In fact, the light-powered splitting of water is responsible for the high amount of oxygen in the atmosphere[177]. When more than two billion years ago free elemental oxygen first appeared in the oceans and subsequently in the atmosphere, the anaerobic organisms where confronted with an increasingly oxidizing environment[178]. Since the

aggressive properties of elemental oxygen and its derivates are a constant source of cell damage, only the development of antioxidant systems including the enzymes superoxide dismutase and catalase allowed the survival under these conditions[179]. Nevertheless, the abundant availability of oxygen led to an enormous improvement of energy metabolism since oxygen can be used as the final electron acceptor during the generation of ATP via oxidative phosphorylation[180]. In fact, most mitochondria containing eukaryotes as well as many bacteria and archaea use this mechanism and moreover, higher eukaryotes are even dependent on this form of ATP generation and therefore must guarantee sufficient oxygen supply. Unicellular as well as less complex organisms can realize this by simple diffusion but higher organisms like vertebrates additionally need a sophisticated respiratory/circulatory system. The oxygen transport in vertebrates is achieved by the bloodstream. For this purpose the blood contains erythrocytes that consist mainly of hemoglobin in order to bind higher amounts of oxygen than normally could be dissolved in the plasma alone. The gradient between inbreathed air and the blood within the capillaries surrounding the alveoli causes the diffusion of oxygen to oxygen depleted red blood cells. The oxygen is released again, when the erythrocytes that are transported by the cardiovascular system reach peripheral tissues. Following the concentration gradient it diffuses to the site of oxidative phosphorylation, the mitochondria.

3.4.1. Oxygen sensing

The carotid body is the organ that monitors the oxygen supply of the mammalian organism as a whole[181]. It is situated near the fork of the common carotid artery and senses not only the oxygen pressure but also the carbon dioxide pressure, the glucose level and the pH value in the blood[182]. The exact mechanisms that are involved in the sensing of oxygen levels by the carotid body are still under investigation and include functions of mitochondria and the cellular membrane of glomus cells. These cells release neurotransmitters that directly induce signaling to the medulla oblongata, the region of the brainstem that regulates oxygen supply relevant parameters like ventilation and heart rate. The carotid body responds to very small changes in oxygen tension of the arterial blood and is very fast in inducing changes of the cardiovascular and respiratory systems. Therefore, it is perfectly adapted to respond to acute changes of oxygen supply induced by increased physical activity.

The cardiovascular system transports oxygen to every tissue of the body but, dependent on variable metabolic activity and the distance of cells to blood vessels, the

oxygen supply of cells varies within the tissues and can even decrease to suboptimal levels. Therefore, each individual cell has certain mechanisms enabling it to sense reduced oxygen levels and result in short/long term adaption to this special situation involving cellular and systemic mechanisms. The sensing systems can be divided into bioenergetic and biosynthetic mechanisms. Bioenergetic mechanisms involve perturbations of the mitochondrial function and the energy state of the cell including the redox state and the change of reactive oxygen species production. Biosynthetic sensing mechanisms include the oxygen dependent function of different enzymes, like NADPH oxidases, heme oxygenases, cytochrome P-450 monooxygenases and 2-oxoglutarate and iron(II)-dependent dioxygenases[183]. The dioxygenases are responsible for the regulation of the transcriptional response to reduced oxygen levels (see below).

3.4.2. Transcriptional response to hypoxia – HIF

The transcriptional response to reduced oxygen supply plays an essential role in the cellular and also in the systemic adaption to hypoxia. The central components of this response in vertebrates are the hypoxia inducible factors (HIFs). HIFs were identified as transcription factors that induce the expression of epo[184] in specialized cells of the kidney and to a lesser extent in the liver and the brain[23,185,186]. Today, more than 100 target genes are known to be directly modulated by the activity of HIFs emphasizing its importance during hypoxia[187]. The transcription factor binds to DNA at the so called hypoxia response element (HRE) that contains an RCGTG consensus sequence. The expression of HIF target genes affects metabolic adaption, erythropoiesis, angiogenesis, vascular tone, cell growth, differentiation, survival and apoptosis[188]. There is also evidence that the HIF-regulated expression of certain microRNAs[189-191] as well as of histone demethylases[192,193] may further broaden the signaling output of the HIF transcription factors.

HIFs are heterodimeric proteins, consisting of an α-subunit that is induced by hypoxia and a β-subunit that is constitutively expressed. Both subunits are basic helix-loop-helix (bHLH) proteins and are part of the PAS domain (Per; AHR; ARNT; SIM) protein family[194]. In mammalians there are three HIF-α proteins that are encoded by distinct gene loci (Figure 5). HIF-1α was the first HIF-α subunit identified and is widely expressed in normal tissues[184]. HIF-2α was identified in endothelial cells but is also expressed in parenchyma and interstitial cells of multiple organs[195,196]. While HIF-1α and HIF-2α share a similar domain structure, HIF-3α is less closely related[197]. Its function is

not well understood, although it has been shown that an alternative splicing variant leading to a shorter version of the protein called inhibitory PAS domain protein (IPAS) forms transcriptionally inactive heterodimers with HIF-1α[198]. This and more recent data indicate that HIF-3α may be of relevance for the regulation and fine tuning of the transcriptional activity of HIFs[199] (Figure 5). In addition to the already mentioned bHLH and PAS domains that are essential for DNA binding and dimerization, HIF-α proteins contain transactivation domains (TAD) that are involved in the recruitment of other transcription factors[200]. During hypoxia, the C-terminal-TAD (C-TAD) is able to interact with the CH-1 domain of the transcriptional coactivator p300/CBP[201]. This interaction is sterically inhibited by the oxygen dependent hydroxylation at an asparaginyl residue[202,203] (see below). Further oxygen dependent regulation is mediated by the two oxygen dependent degradation domains (N-terminal, N-ODD and C-terminal, C-ODD)[204,205], each of which contains a prolyl residue that is hydroxylated if oxygen is present[206,207]. The hydroxylated prolyl residues trigger the interaction with the von Hippel-Lindau tumor suppressor protein (pVHL) that is part of an E3 ubiquitin ligase complex which ubiquitinates the HIF-α proteins at several sites leading to proteasomal degradation[208]. Additionally, several other factors influencing the activity of HIFs besides hydroxylation are known to date, but their physiological relevance is still under discussion[209].

The HIF-1β proteins, also known as aryl hydrocarbon receptor nuclear translocators (ARNT1, 2 and 3) contain a bHLH and a PAS domain but lack the ODDs making them insensitive to oxygen supply[194,210,211]. HIF-1β forms heterodimers with HIF-α proteins as soon as the latter are stabilized under hypoxic conditions.

Knock out studies have shown that HIF-1α as well as HIF-2α are both essential for normal embryogenesis. Interestingly, inactivation of either one of the two proteins leads to different phenotypes, indicating their non redundant functions in the organism. HIF-1α negative mice die around E10.5 and these embryos show defective vascularization and cardiac morphogenesis[212]. Furthermore, heterozygous or tissue specific loss of HIF-1α function has revealed the importance of this transcription factor for hypoxic adaption and also for physiological functions in different cell types as well as for tumor survival[188,213-215]. The phenotype of HIF-2α inactivation has been shown to be dependent on the genetic background. Whereas the first established knock out mice died in utero (E9.5-13.5) due to defective catecholamine production and vascularization[216,217] backcrossing into different mouse strains resulted in a small number of viable adult mice that suffer from multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species[218]. The distinct phenotypes of HIF-1α and HIF-2α deficient mice demonstrated that the two transcription factors have unique targets.

Further studies that addressed the question of specific transcriptional activation in more detail revealed that HIF-1α is responsible for the hypoxic dependent regulation of several glycolytic enzymes, carbonic anhydrase and BNIP-3[219-221] whereas HIF-2α induces the expression of Oct4, cyclin D1, TWIST1, TGF-α and epo amongst others[222-226]. Finally there are also genes like VEGF, adrenomedullin and GLUT-1 that are regulated by both transcription factors[219,227]. In general, HIF-1α and HIF-2α bind to HREs equally well and consequently this does not play a major role in specificity of transcriptional induction[228]. In fact, it could be shown that the C-terminal domains, which mediate the interaction with other transcription factors, are responsible for the target specificity of HIFs[229,230]. Further investigation is needed to fully understand the mechanisms determining the distinct targets of both HIFs that may include interaction with other transcription factors, signaling cascades and the local chromatin status.

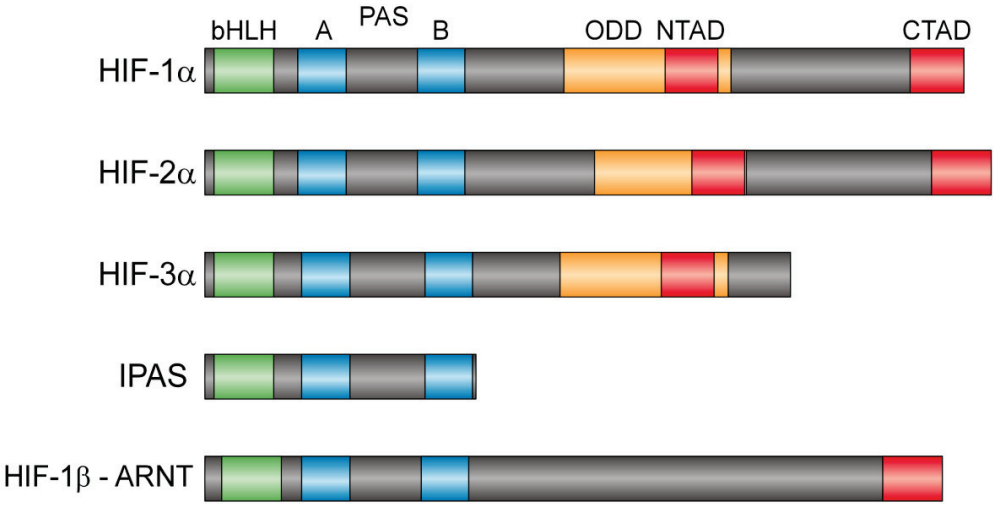

Figure 5. Domain structure of HIF family members. All HIF proteins contain a bHLH domain needed for DNA binding and two PAS domains involved in dimer-formation. The HIF-α subunits additionally contain an oxygen dependent degradation domain (ODD) making these proteins sensitive to high oxygen levels. The initial signal for HIF-1α degradation is the hydroxylation of two proline residues of this domain. Within the ODD lies the N-terminal activation domain (NTAD) that is involved in the interaction with other transcription factors. The C-terminal activation domain (CTAD) of HIF-1α and HIF-2α contains an asparaginyl residue that is hydroxylated as well when oxygen is available, inhibiting the interaction of the CTAD with transcription factors like p300. IPAS is a splice variant of HIF-3α and is assumed to be involved in the regulation of HIF activity. HIF-1β or ARNT lacks the ODD and therefore is insensitive to oxygen dependent degradation. Together with the HIF-α subunits HIF-1β forms heterodimers, i.e. functional HIF transcription factors. Figure adapted from Lisy and Peet, Cell Death and Differentiation, 2008[231].

3.4.3. Regulation of HIF-α by oxygenases – PHDs and FIH1

As already mentioned above, the oxygen dependent hydroxylation of a specific asparagyl residue interferes with the interaction of the HIF-α C-TAD with p300/CBP, thereby reducing its activity as transcription factor. Furthermore, hydroxylation of either one or both of two prolyl residues triggers the proteasomal degradation of HIF-α by promoting pVHL dependent ubiquitination (Figure 6). Both modifications are catalyzed by hydroxylases that belong to the family of 2-oxoglutarate and iron(II)-dependent dioxygenases[231,232]. As indicated by the name, the activity of these enzymes depends on the availability of ferrous iron that is situated in the enzymatic center of the proteins[233,234]. There it is involved in the transfer of one oxygen atom of O_2 to the target amino acid whereas the second atom is transferred to 2-oxoglutarate, which leads to the formation of succinate and CO_2. Therefore, besides low oxygen levels, chelating or replacing iron by other metal ions like cobalt as well as the replacement of 2-oxoglutarate by structural homologues like dimethyloxalylglycine (DMOG) inhibit oxidase activity and consequently stabilize and activate HIFs[207,235].

The two proline residues are hydroxylated by HIF prolyl hydroxylases (PHDs) that belong to the larger family of prolyl-4-hydroxylases[236]. In mammalians there are three isoforms described: PHD1-3[237]. PHD2 is the most abundant isoform whereas PHD1 and PHD3 expression is restricted to specific tissues like testis and heart[238]. Moreover, PHD2 prefers HIF-1α as substrate, while PHD1 and PHD3 prefer HIF-2α[239]. Differences in tissue distribution and different contribution to the regulation of the two HIFs may therefore partly explain the observed target specificity of HIF-1α and HIF-2α. Recently, P4H-TM, a prolyl-4-hydroxylase that is located in the endoplasmatic reticulum membrane, has been shown to hydroxylate HIFs as well but its physiological importance for the regulation of HIFs is still under investigation[240].

In contrast to prolyl hydroxylation, asparaginyl hydroxylation is performed by one single enzyme called factor inhibiting HIF (FIH)[202,241,242]. FIH is ubiquitously expressed and predominantly localized in the cytoplasm[243,244]. Interestingly, *in vitro* data showed that the K_M values of PHDs for oxygen are higher than those of FIH indicating that FIH is still active when prolyl hydroxylation is already absent due to reduced oxygen levels[245,246]. Therefore, in a mild hypoxic environment C-TAD mediated transcriptional activation by p300/CBP is still blocked although the HIF proteins are already stabilized and the N-TAD is active[231]. Generally, the K_M values for oxygen of all hydroxylases involved in HIF-α regulation are relatively high compared to physiological oxygen concentrations. Hence, the change of hydroxylase activity is highly dynamic over the

whole physiological range of oxygen concentrations and allows immediate reaction to even very small changes of oxygen supply[183,247,248].

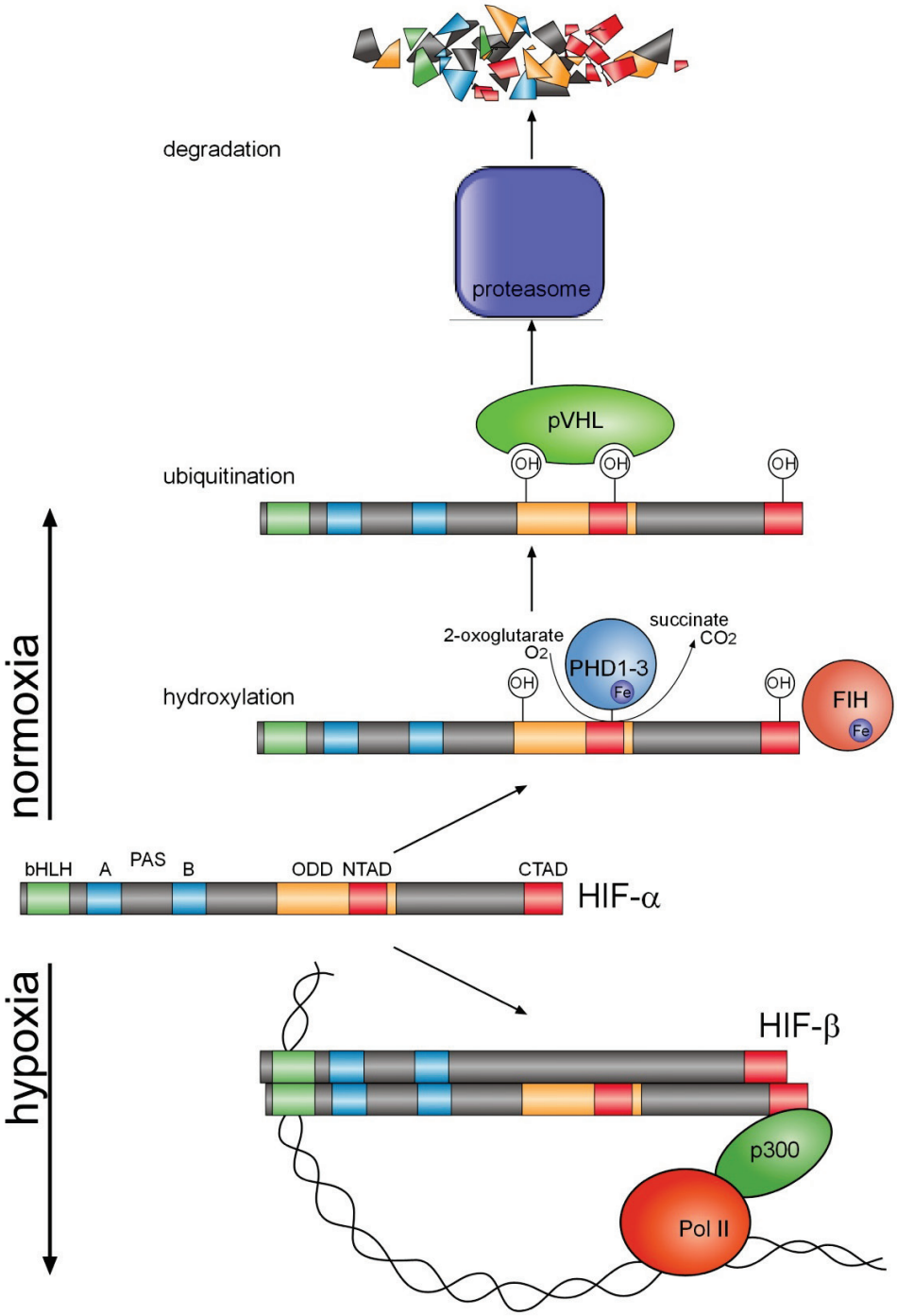

Figure 6. Regulation of HIF activity. Under hypoxic conditions HIF-α subunits form dimers with HIF-β subunits and induce the transcription of HRE regulated genes by the interaction with other TFs like p300. However, if oxygen is available, HIFs are hydroxylated by FIH and PHD1-3, which leads to transcriptional inactivation and eventually ubiquitination of the protein. The ubiquitinated HIF-α proteins are recognized and degraded by the proteasomal complex. See text for more details.

3.4.4. Oxygen distribution in the mammalian body

In order to provide all cells of the body with oxygen, higher organisms have developed sophisticated transport systems using convection as the driving force. In the case of mammalians this system consists of the respiratory tract and the cardio-vascular system. Nevertheless, the oxygen supply is also dependent on diffusion that allows the transition of oxygen through the alveolar-capillary membranes as well as from the blood to mitochondria, the main oxygen consumers. Due to the cell type dependent equilibrium between delivery and consumption the oxygen concentration of different tissues as well as within a tissue itself shows considerable variation[183]. The lung parenchyma are obviously relatively well supplied with oxygen due to the direct contact to atmospheric oxygen levels of 20-21%[249]. Accordingly, the arterial blood that leaves the lungs still contains about 14% oxygen but the oxygen concentration steadily decreases to about 4% when it reaches the capillaries of the most peripheral tissues[250,251]. Therefore, the range of oxygen concentration within organs that are well supplied with arterial blood like the liver and the heart are comparable to the concentrations measured within the blood itself. However, the combination of constant oxygen consumption and increased distance to the capillaries leads to a lower oxygen concentration at least in some regions especially of less well irrigated tissues. Measurements of the brain oxygenation showed that depending on the observed region, the oxygen concentration lies between 0.5% and 7%[252,253]. Comparable results could be obtained for the bone marrow although the levels here are even lower, lying between 0% and 5%[254,255]. Chow et al. further addressed the question of oxygen distribution in the bone marrow based on the Kroghian model in more detail[256-259]. Founded on their calculations they could show that hematopoietic progenitors are localized in regions of the bone marrow with very low oxygen tension. Furthermore, the oxygen concentration within smaller structures like the erythroblastic island becomes reduced towards the center and increased maturity of the erythroblasts correlates with increased oxygen tension. This correlation of tissue oxygen concentration and maturity of hematopoietic cells doesn't seem to be a coincidence but can contribute to the decision between self renewal and differentiation. In fact, it has been shown that low oxygen levels support the maintenance of stem cells and enhance the self renewal capacity of early progenitor cells of several lineages including erythroblasts[260-265]. The underlying mechanisms are still unknown and may be linked to the reduced formation of ROS or the hypoxia induced expression of certain growth factors and cytokines[266].

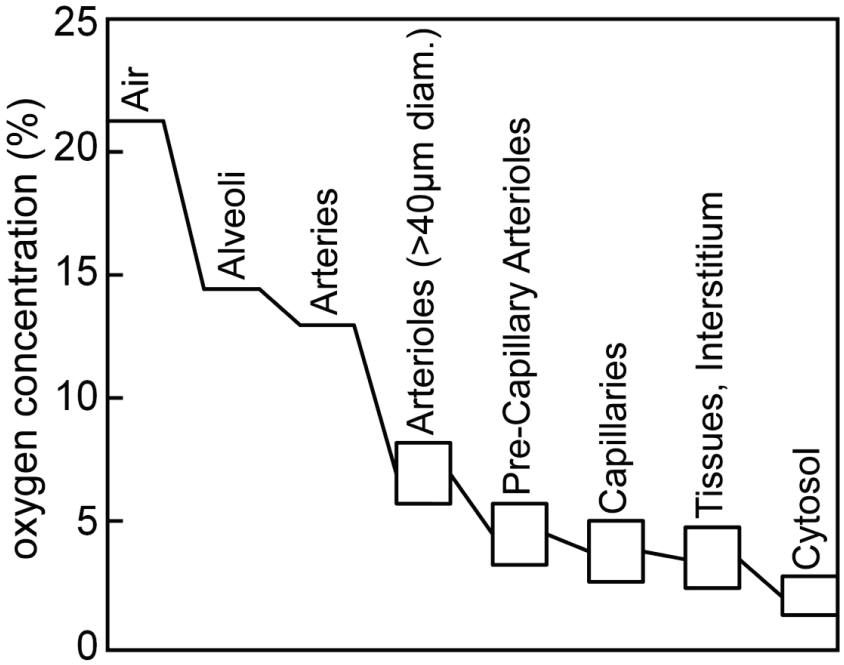

Figure 7. Distribution of oxygen concentrations in the cardiovascular system and tissues. The rectangles represent variation of the available data. Note that in some cases the oxygen concentration in tissues and the cytosol is lower and can even drop to zero. For further details see text. Figure simplified from Ward, Biochimica et Biophysica Acta 2008[183].

3.4.5. Oxygen and tissue culture

As mentioned above, the oxygen concentration plays an important role in the fate of cells *in vivo*. During the *in vitro* culture of cell lines or primary cells this is of equal relevance. In fact, it is crucial for the work with *in vitro* cultures to establish conditions that resemble the physiological situation, since inadequate oxygen supply may affect the cultivation of the cultivated cells in general and in addition, may influence the specific mechanisms that are analyzed. Nevertheless, the standard tissue culture incubator does not allow the adjustment of the oxygen concentration but uses the ambient atmosphere containing 20-21% oxygen supplemented with CO_2. In addition, the use of conventional polystyrene dishes in tissue culture limits the oxygen supply of the cells by one dimensional diffusion through the culture medium. This is physically described by Fick's first law of gas diffusion that adapted to tissue culture conditions says that:

$$\frac{dUoxy}{dt} = -DA\frac{Cgas - Ccell}{h}$$

where dUoxy/dt is the quantity of oxygen transported from the top to the bottom of the culture dish in a period of time, D is the diffusion constant of the medium, A is the area of diffusion, C_{gas} is the oxygen concentration defined by the composition of the incubator atmosphere, C_{cell} is the oxygen concentration at the bottom of the dish (i.e. pericellular oxygen concentration) and h is the diffusion distance from the top to the bottom of the dish[267]. Several papers that addressed the problem of oxygen supply in tissue culture on the basis of this equation came to the conclusion that several cell types that are cultivated at 20% oxygen under conventional conditions are indeed facing a severely hypoxic environment since the oxygen consumption rate exceeds the diffusion rate through the culture medium[268-270]. Metzen et al. confirmed these estimations by comparing the calculations with direct measurements of the pericellular oxygen concentration[271]. Despite these observations, some cell culture studies that address oxygen dependent mechanisms are performed at reduced levels of ambient oxygen. These levels were chosen to resemble the oxygen concentration in the corresponding tissue in order to ensure physiological conditions. In view of the fact that atmospheric oxygen levels are often insufficient to allow adequate oxygen supply in tissue culture, further reduction of the oxygen concentration rather leads to the establishment of severely hypoxic conditions that eventually result in artificial results. Thus, tissue culture experiments must be performed under consideration of the cell type, the state of confluence, the metabolic activity, the diffusion distance and the oxygen fraction of the incubator atmosphere in order to ensure reliable results[267].

4. Aims of this work

Although erythropoiesis is a well studied process there are still several open questions to be addressed. The formation of red blood cells is dependent on the import and handling of exceptionally increased amounts of iron but since an excess of iron is toxic this requires stringent regulation. In non erythroid cells this is perfectly accomplished by the IRE/IRP system. Nevertheless, with erythroid cells the situation is more complex. IRP binding activity regulates the equilibrium between iron import by TfR1 and iron storage by ferritin[47]. During terminal erythroid differentiation iron accumulates within the cell, which conventionally leads to a decrease of iron import and increased storage of iron within ferritin shells and would interfere with normal heme synthesis. An additional aspect that contributes to the complexity of the situation is the IRP dependent posttranscriptional regulation of the erythroid specific enzyme ALAS2. ALAS2 and ferritin contain both an IRE within their 5'UTR indicating synchronous posttranscriptional regulation. Nevertheless, during the period of heme synthesis the expression of ferritin needs to be low in order to prevent iron sequestration while the increased expression of ALAS2 is essential for protoporphyrin synthesis. We addressed the question how erythroid cells accomplish to maintain sufficient iron supply during differentiation using an erythroid culture system that is perfectly suited to analyze system intrinsic processes under *in vivo*-like conditions[43].

The analysis of IRP1 and IRP2 deficient mice has shown that the lack of IRP1 does not affect erythropoiesis whereas IRP2 knock out mice show microcytic anemia, at least under laboratory conditions[167,168]. Since the targeted deletion of IRP2 in macrophages, hepatocytes and enterocytes shows no erythroid related phenotype, this indicates that the observed anemia of IRP2 deficient mice is an inherent erythroid phenotype[172]. We were interested in the specific contribution of IRP1 and IRP2 on the regulation of ferritin and TfR1 expression in our erythroid culture system that allows the analysis of the anemic phenotype in more detail. Hence, with this system we are not only able to modulate heme synthesis and the iron household of the cells but can also monitor the differentiation of IRP1 and IRP2 deficient erythroblasts in a defined environment, making it possible to investigate the intrinsic effects of disturbed IRP activity.

The oxygen concentration is known to influence the binding activity of IRPs as well as the process of erythroid differentiation. The latter is not only affected indirectly by hypoxia dependent expression of epo but also directly by a still less well understood

mechanism. Nevertheless, the classical cultivation of cells within incubators does not consider the adjustment of the oxygen concentration to physiological levels. In order to minimize artificial effects on the outcome of our work due to inappropriate oxygen supply we were interested in the influence of changed oxygen concentration on the iron regulatory system as well as on the proliferation and differentiation of cultivated erythroblasts. Furthermore, we addressed the question, which mechanism is relevant for the direct influence of oxygen on erythropoiesis.

5. Results - Manuscripts

The data presented in the result section are

5.1. Iron metabolism in primary mouse erythroblasts: Differentiating cells bypass the IRP system to ensure heme biosynthesis

Matthias Schranzhofer, **Manfred Schifrer**, Javier Antonio Cabrera, Hartmut Beug, and Ernst W. Muelllner

published in Blood 2006, May 15;107(10):4159-67.

Manfred Schifrer contributed to the generation of the data, the discussion of the results and the writing of the paper.

5.2. Low cytosolic non-heme iron levels in erythroid cells prevent IRP2-mediated ferritin upregulation during differentiation

Matthias Schranzhofer, **Manfred Schifrer**, Bruno Galy, Matthias Hentze, Ernst W. Muellner, and Prem Ponka

prepared for submission to Blood

Manfred Schifrer generated the data presented in figure 1, 3, 4 and contributed to data shown in figure 5, to the discussion of the data and to the writing of the manuscript.

5.3. Oxygen directly regulates terminal erythroid differentiation by modulating the activity of 2-oxoglutarate dependent oxygenases

Manfred Schifrer, Matthias Schranzhofer, Hartmut Beug, and Ernst W. Muellner

prepared for submission to Haematologica

Manfred Schifrer designed and performed the experiments and wrote the manuscript.

5.1. Iron metabolism in primary mouse erythroblasts: Differentiating cells bypass the IRP system to ensure heme biosynthesis

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5.1.1. Abstract

Terminal erythropoiesis is accompanied by extreme demand for iron to ensure proper hemoglobinization. Thus, erythroblasts must modify the "standard" post-transcriptional feedback regulation, balancing expression of ferritin (Fer; iron storage) versus transferrin receptor (TfR1; iron uptake) via specific mRNA binding of iron regulatory proteins (IRPs). Although erythroid differentiation involves high levels of incoming iron, TfR1 mRNA stability must be sustained and Fer mRNA translation must not be activated because iron storage would counteract hemoglobinization. Furthermore, translation of the erythroid-specific form of aminolevulinic acid synthase (ALAS-E) mRNA, catalyzing the first step of heme biosynthesis and regulated similarly as Fer mRNA by IRPs, must be ensured. We addressed these questions using mass cultures of primary murine erythroid progenitors from fetal liver, either undergoing sustained proliferation or highly synchronous differentiation. We indeed observed strong inhibition of Fer mRNA translation and efficient ALAS-E mRNA translation in differentiating erythroblasts. Moreover, in contrast to self-renewing cells, TfR1 stability and IRP mRNA binding were no longer modulated by iron supply. These and additional data stemming from inhibition of heme synthesis with succinylacetone or from iron overload suggest that highly efficient utilization of iron in mitochondrial heme synthesis during normal erythropoiesis alters the regulation of iron metabolism via the IRE/IRP system.

5.1.2. Introduction

Maturing erythroid progenitors require large amounts of iron to enable high rates of heme synthesis. The daily generation of about 20 g erythrocytes in adult humans requires the delivery of 20 mg iron via iron-loaded transferrin (Fe_2-Tf) to the bone marrow. The corresponding transferrin receptor (TfR1) is highly expressed on the cell surface of erythroblasts. Recently, a second variant, TfR2, was characterized, which among other cells is also found on the surface of immature erythroid cells[1,2], but seems to play a minor role during terminal maturation[2,3]. Tf-TfR1 complexes are internalized into endosomes via receptor-mediated endocytosis, and endosome acidification leads to release of iron[4,5] and, subsequently, to its export into the cytoplasm via the transporter DMT-1[6,7]. Finally, iron is delivered to its sites of utilization; any excess gets stored in ferritin (Fer). This sequestration is important to avoid oxidative damage via this highly reactive metal. Therefore, the balance between iron uptake, utilization, and storage has to be tightly regulated.

Cis-acting elements involved in this control are the stem-loop structures called iron regulatory elements (IREs) in several mRNAs (for a review, see Pantopoulos[8]). Such IREs are found in the 5'-untranslated region (UTR) of ferritin light (FerL) and heavy chain (FerH) mRNA, and in 5 copies within the 3'-UTR of TfR1 mRNA. These elements are recognized by *trans*-acting factors, that is, iron regulatory proteins 1 and 2 (IRP1, IRP2), which bind with high affinity to IREs in their cognate mRNAs. One difference between IRP1 and IRP2 lies in their mode of regulation. IRP1 is a bifunctional protein, which under high iron releases the IRE, incorporates a cubane 4Fe-4S cluster, and gains enzymatic activity as cytosolic aconitase[9]. In contrast, IRP2 mRNA-binding activity is turned off by proteasomal degradation[10-12].

In a generally accepted "standard" model, cytosolic iron concentrations regulate the mRNA-binding activity of IRP1/2 to IREs, which are localized in the UTRs of a growing number of transcripts[8]. IRPs bind to IREs under low iron concentrations, which, for example, inhibits translation of Fer messages[13,14] and stabilizes TfR1 mRNA[15-17]. Consequently, cellular iron uptake is stimulated[18,19], whereas storage is inhibited. At high iron concentrations, IRP1 incorporates iron-sulfur clusters and thus does not bind target mRNAs while IRP2 gets degraded[10-12]. This leads to an increase in Fer synthesis and TfR1 mRNA degradation via a specific endonuclease pathway[15-17], which in turn reduces

cellular iron uptake rates. These posttranscriptional feedback mechanisms allow cells to balance cellular iron homeostasis.

There are, however, growing numbers of reports on specialized cell types and tissues that at least under certain instances bypass the IRE/IRP system[20-23], one prominent example being erythroid cells[24,25]. Apart from their especially high iron needs, they express an erythroid-specific isoform of aminolevulinic acid synthase (ALAS-E), a key enzyme in erythroid heme synthesis. ALAS-E is essential for erythroid differentiation[26]. Like Fer mRNAs it contains a functional IRE in its 5'-UTR (for a review, see Sadlon et al[27]). Thus iron-dependent regulation of ALAS-E should allow cells to coordinate the production of protoporphyrin IX with cellular iron levels. The resulting coregulation of TfR1, Fer subunits, and ALAS-E may be meaningful in erythroid cells undergoing self-renewal (ie, sustained proliferation without differentiation), but once induced for terminal differentiation, these cells need saturating doses of Fe_2-Tf for proper maturation[28-30]. Consequently, committed erythroblasts have to maintain high expression of TfR1 despite increasing intracellular iron concentrations. Furthermore, synthesis of Fer must not be activated by incoming iron, because this would lead to counterproductive storage in a phase of high iron demand[31]. Thus, the regulation of TfR1, Fer, and ALAS-E expression during erythroid differentiation is difficult to reconcile mechanistically with the principles outlined.

In this paper, we therefore addressed iron metabolism during terminal erythropoiesis of primary mouse erythroid progenitors, using a unique hematopoietic culture system. This, for the first time, permitted us to analyze the complexity of posttranscriptional regulation of IRE-containing mRNAs by iron during sustained proliferation versus terminal differentiation of immature erythroid progenitors under in vivo–like conditions. This cell model allows (1) proliferation and differentiation of erythroblasts derived from mouse fetal liver in serum-free medium, (2) mass cultures with up to 10^7-fold expansion of cells, yielding enough material for studies requiring a high amount of sample material, such as polysome gradient analyses, and (3) production of enucleated, fully hemoglobinized cells[32,33].

Analyses of self-renewing or differentiating mouse erythroblasts demonstrated that the coordinate regulation of Fer, ALAS-E, and TfR1 mRNAs via iron is abolished during terminal differentiation. Instead, both proteins involved in iron homeostasis (Fer and TfR1) are insensitive toward changes in physiologic concentrations of Fe_2-Tf. Moreover, translation of Fer mRNA is almost entirely blocked, whereas ALAS-E mRNA is used to a significant extent. Artificially boosting cytosolic iron levels by addition of ferric

ammonium citrate (FAC), succinylacetone (SA), an inhibitor of heme biosynthesis, or unphysiologically high concentrations of Fe_2-Tf could, however, reinduce expression of Fer in maturing erythrocytes.

5.1.3. Material and Methods

Culture of primary mouse erythroblasts

Erythroid cells were isolated and cultivated as described[32,33]. Briefly, cells were grown from fetal livers from E12.5 embryos (wild-type, MF1 background) and resuspended in serum-free StemPro-34 medium plus Nutrient Supplement (Invitrogen-Gibco, Carlsbad, CA) plus 2 U/mL human recombinant erythropoietin (Epo; 100 ng/mL), murine recombinant stem cell factor (SCF; 100 ng/mL), the synthetic glucocorticoid dexamethasone (Dex; 10^{-6} M), and insulin-like growth factor 1 (IGF-1; 40 ng/mL). Cell number and size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany). Dead or differentiating cells were removed by Ficoll purification.

To induce terminal differentiation, continuously self-renewing erythroblasts were washed twice in PBS and seeded in StemPro-34, containing 10 U/mL Epo, insulin (4 x 10^{-4} IU/mL), the Dex antagonist ZK-112993 (3 x 10^{-6}M)[34] and iron-saturated human transferrin (Fe_2-Tf; 1 mg/mL = 12.5 µM = 25 µM Fe = physiologic levels; Sigma, St Louis, MO). Where indicated, heme synthesis was inhibited by 0.2 mM SA (Sigma)[35].

To induce iron starvation, cells were incubated with 50 µM of the iron chelator desferrioxamine (Des); iron overload was induced by adding Fe_2-Tf up to 0.1 mM or FAC (20 µg/mL, 17% saturation = 63 µM iron) 24 hours before harvest.

Cell morphology, histologic staining, and determination of hemoglobin content

Changes in cell morphology during differentiation were monitored by phase-contrast microscopy. For histologic analysis, erythroblasts were cytocentrifuged[36] at various stages of maturation onto glass slides and stained with histologic dyes and neutral benzidine for hemoglobin as described[36]. Hemoglobin content was analyzed by removing 50-µL aliquots from the cultures and by undergoing photometric determination as described[37]. Values obtained from triplicate determinations were averaged and normalized to cell number and cell volume.

Flow cytometry

Self-renewing or differentiating erythroblasts (1 x 10^6) were washed twice with PBS/2% fetal calf serum (FCS) and stained with fluorescently labeled antibodies against transferrin receptor (FITC; PharMingen, San Diego, CA; no. 01595) and Ter119 (PE; PharMingen, no. 09085)[38]. Surface marker expression was analyzed by flow cytometry (LSR-I; Becton Dickinson, Franklin Lakes, NJ).

Northern blot analysis

Total RNA was prepared from 2 to 4 x 10^7 cells using TRIzol (Invitrogen). Then, 10 µg total or polysomal RNA/sample was separated in denaturing 1% formaldehyde-agarose gels. Equal loading was controlled by ethidium bromide staining. RNA was transferred to nylon membranes (Gene Screen; DuPont, Wilmington, DE), and fixed by UV irradiation (1200 mJ; UV-Crosslinker; Stratagene, LaJolla, CA). Membranes were sequentially hybridized with [^{32}P]-labeled cDNA probes generated by random-primed labeling (Prime-it-II; Stratagene) specific for mouse FerH (0.85-kb *Aat*II-*Nde*I fragment), FerL (0.3 kb, *Aat*II-*Nde*I), ALAS-E (1.4 kb, *Aat*II-*Not*I), TfR1 (0.75 kb, *Eco*RI-*Hin*dIII), and α-globin mRNAs. Probes for IRP1 and IRP2 mRNA were obtained from the RZPD library of the German Resource Center for Genome Research (#IMAGp998J182131, *Eco*-*Hin*dIII; IRP2 #IMAGp998I191339, *Sac*II-*Mlu*I, respectively). Signals were quantified by phospho-imaging.

Polysome gradients

The extent of mRNA association with polysomes was determined by sucrose-gradient analysis[39] with RNA prepared from 2 to 4 x 10^7 cells. After removal of nuclei and cell debris by centrifugation, lysates were laid onto 15% to 40% sucrose gradients and separated by ultracentrifugation. RNA was harvested from 18 fractions, separated on denaturing agarose gels, and transferred onto nylon membranes as described[40]. Distribution of 18S and 28S rRNA was visualized by staining of filters with methylene blue.

Electrophoretic mobility shift assays

RNA-protein complexes were resolved essentially as described[39,41]. Briefly, for electrophoretic mobility shift assays (EMSAs), cytoplasmic extracts were incubated with [^{32}P]-labeled transcripts produced by T7 RNA-polymerase after linearization of the plasmid pGEM-3Zf(+)-mouse FerH-IRE (clone42)[42] with *Bam*HI. Protein (2 µg) and 1.3 x 10^6 disintegrations per minute of labeled IRE-containing in vitro transcript were incubated for 20 minutes at room temperature. The total amount of IRP1 was assessed by in vitro reduction with 2% β-mercaptoethanol (2-ME)[43] prior to the binding reaction. After treatment with RNAse T1 and heparin, RNA-protein complexes were resolved on 6% non–denaturing polyacrylamide gels at 4°C. Bands corresponding to IRE/IRP complexes were quantified by phospho-imaging (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis

Cell pellets were lysed in sample buffer as described[38] and 10 to 20 µg protein was separated on sodium dodecyl sulfate-polyacrylamide gels. Protein transfer and loading were visualized by staining with acidic Ponceau-S solution. Thereafter, membranes were blocked 1 hour at room temperature with 1% low-fat dry milk in TBS and probed overnight with anti–horse spleen ferritin (Sigma; no. F-6136) or anti–rat IRP1[44], rat monoclonal anti–mouse TfR1 (BioSource, Nivelles, Belgium; no. AMS7102) or, for normalization, with anti-Erk1/2 (Sigma; no. 5670). After washing, filters were incubated with second antibody (horseradish peroxidase–coupled anti–rabbit IgG antibody (Jackson Laboratories, West Grove, PA; no. 111-035-008) for Fer, IRP1, Erk1/2, eIF4E, and anti–rat IgG (Jackson Laboratories; no. 112-035-008) for TfR1. After washing, immunoreactive signals were detected by enhanced chemoluminescence (Amersham, Buckinghamshire, United Kingdom).

ALAS-E immunoprecipitation and enzyme activity

Protein levels of ALAS-E were determined by immunoprecipitation of [^{35}S]-methionine pulse-labeled cell extracts using a polyclonal antiserum developed in rabbit (a kind gift from M. Hentze and B. Galy, European Molecular Biology Laboratory, Heidelberg, Germany EMBL) and visualized by phospho-imaging. Enzyme activity was determined

by fluorometric high-performance liquid chromatography (HPLC) of the reaction product δ-aminolevulinic acid[45] in cell extracts after removal of nuclei.

5.1.4. Results

Extended self-renewal and synchronous differentiation of primary, fetal liver–derived erythroblasts

Most studies on iron metabolism in erythroid cells so far were done with erythroleukemic cell lines or reticulocytes. Murine erythroleukemia cells have provided insights into mechanisms controlling erythroid differentiation[46] but have severe drawbacks. Importantly, they are unresponsive toward physiologic maturation stimuli, including Epo. Thus, differentiation is induced artificially by nonphysiologic agents such as dimethyl sulfoxide, hexamethylenebis-actamid and the like. In addition, poor hemoglobinization and abnormal morphologic changes during differentiation originate from patterns of gene expression different from those in normal erythropoiesis[47]. Reticulocytes represent a more physiologic system, but poorly represent the proliferative and early differentiation aspects of erythropoiesis[48].

In this study, primary mouse erythroid cells were used, which closely recapitulate several aspects of terminal maturation in vivo, including size decrease, full hemoglobinization, and enucleation[33,49]. These cells, from fetal livers of E12.5 mouse embryos, can be expanded under self-renewing conditions (sustained proliferation without differentiation) for 15 to 20 days before undergoing senescence (Figure 1A). Substitution of SCF plus Dex with insulin and the glucocorticoid antagonist ZK[34] plus increase of erythropoietin are sufficient to induce highly synchronous terminal maturation, largely completed within 72 hours (Figure 1B). Noteworthy, the most substantial increase in hemoglobin content occurs between 24 and 48 hours after induction of differentiation (Figure 1D), accompanied by cell size decrease to half the original volume (Figure 1C). At this stage, the erythroblasts up-regulate differentiation markers like Ter119, whereas markers typical for immature cells disappear[32]. Thus, with respect to regulation of iron metabolism, special focus was put on this interval. This period is also characterized by sufficient transcriptional activity allowing interference with gene expression, whereas at later stages chromatin condensation (prior to enucleation) shuts down nuclear activity.

Translational repression of Fer mRNA and efficient utilization of ALAS-E mRNA in differentiating mouse erythroblasts

Following the "standard" model of IRP/IRE-mediated translational regulation, increase in cellular iron uptake in hemoglobinizing cells should not only activate more efficient translation of ALAS-E mRNA (to ensure high levels of protoporphyrin IX synthesis) but also favor (futile) synthesis of iron storage proteins.

To address this question, translation of both Fer and ALAS-E mRNAs was monitored by polysome gradient analysis of erythroblasts kept under proliferation or differentiation conditions, either iron-depleted by addition of the iron chelator Des or fully iron-loaded by incubation with additional Fe_2-Tf (1 mg/mL). Polysome-associated mRNAs were fractionated from untranslated mRNPs by linear sucrose gradients (see "Materials and methods"). After RNA isolation and separation, blots were hybridized with probes specific for FerH, FerL, ALAS-E, and α-globin mRNAs. In self-renewing erythroblasts, translation of FerH, FerL, and ALAS-E mRNAs was still modulated by availability of iron (Figure 2A). Under high iron conditions, there was an increase of mRNA in polysome-bound fractions from 2% to 9% for FerH, from 2% to 19% for FerL, and from 9% to 25% for ALAS-E (Figure 2B, left panels). The small percentages of polysome-bound FerL/H mRNA in proliferating erythroblasts even under high iron conditions are comparable to the earlier observations[51,52]. When erythroblasts differentiating for 48 hours were subjected to the same analysis, however, iron-induced translational activation of FerH or FerL (or both) was abolished (Figure 2A-B). Control α-globin mRNA was translated with high efficiency under both low and high iron conditions. Thus, Fer mRNA translation can be activated by physiologic concentrations of Fe_2-Tf under self-renewal conditions but not in maturing erythroblasts that are accumulating hemoglobin. In contrast, ALAS-E mRNA translation remained regulated by iron levels after the onset of differentiation (16% polysome-bound mRNA under iron starvation versus 41%; Figure 2B, right panels). Therefore, under all conditions tested, ALAS-E mRNA was translated more efficiently than Fer transcripts in proliferating as well as differentiating cells.

In maturing erythroid progenitors, efficient ALAS-E mRNA translation was accompanied by a massive increase in transcript levels of more than 20-fold. Interestingly, also transcription of FerL augmented significantly (3-fold; Figure 2E)[53] but unlike ALAS-E mRNA, this increase was not accompanied by an increase of transcripts engaged to polysomes (Figure 2B), as reported earlier for differentiating murine erythroleukemia (MEL) cells[54].

The data on the mRNA level were corroborated by analyses at the protein level. In differentiating mouse erythroblasts Fer expression stayed at low levels, irrespective of cell preincubation with Des or Fe_2-Tf (Figure 2C), whereas ALAS-E protein synthesis rates as measured by immunoprecipitation indicated a 2.4-fold increase (Figure 2D). The low levels of ALAS-E protein synthesis in proliferating cells were in accordance with the much lower abundance of the corresponding mRNA (Figure 2E). Moreover, quantitation of the ALAS-E reaction product, δ-aminolevulinic acid by HPLC produced similar results (data not shown).

TfR1 expression in differentiating primary mouse erythroblasts is independent of iron

It is well documented that during definitive erythropoiesis uptake of iron by mouse erythroid cells is mediated predominantly via Fe_2-Tf/TfR1 endocytosis[30]. Studies with mouse erythroid cell lines[24,25] have emphasized the importance of this pathway to supply maturing erythroid cells with sufficient iron. After initial work with primary chicken erythroid progenitors[24,55], here we extended these observations to primary mouse erythroblasts, using physiologic concentrations of Fe_2-Tf as iron source and Des to induce iron deprivation. Whereas in self-renewing cells TfR1 mRNA levels were regulated by iron, erythroblasts induced to differentiate maintained high expression of TfR1 mRNA under both conditions (Figure 3A). Furthermore, TfR transcript levels in maturing cells were elevated, even in comparison to self-renewing cells supplemented with Des. The corresponding total cellular TfR1 protein levels followed the same pattern (Figure 3B). Additionally, cell-surface TfR1 expression was determined by flow cytometry. In the absence of iron chelator, self-renewing erythroblasts decreased the number of TfR1 molecules on the cell surface, whereas during differentiation, in the phase of high iron demand, no iron-dependent changes could be observed (Figure 3C). Interestingly, although terminal erythropoiesis led to a significant elevation of TfR1 mRNA and total protein levels, cell-surface expression was even somewhat reduced compared with self-renewing cells, reminiscent of the situation in chicken erythroblasts[55] and arguing for redistribution of TfR1 toward later endosomal compartments in the cell during maturation[56].

In differentiating erythroblasts, IRP1 and IRP2 are not regulated by iron

Next we sought to address the role of IRPs during self-renewal and late stage of erythroid differentiation. For this, we performed EMSAs between IRP1 and IRP2 and radiolabeled mouse FerH-IRE RNA probes transcribed in vitro, using the IRE probe C42, which was shown to exhibit an equal binding affinity for IRP1 and IRP2.[42]

In line with the regulation observed for FerH/L, ALAS-E, and TfR1 mRNAs, self-renewing erythroblasts still showed an iron-dependent regulation of IRP1 mRNA-binding activity, even though this difference was clearly weaker compared with extracts prepared from control mouse embryo fibroblasts (Figure 4A). In differentiating erythroblasts, however, mRNA-binding activity of IRP1 declined strongly and remained totally indifferent toward iron sequestration. To determine the total amount of potentially available IRP1 mRNA-binding capacity, β-mercaptoethanol (2-ME)[43,57] was added to the binding reaction prior to addition of labeled IRE probes. Whereas this treatment strongly activated binding activity in self-renewing erythroblasts as well as control cells, the level of activation in differentiating cells was weaker, although comparison of the differences in IRP1 expression revealed that total IRP1 protein and mRNA levels from self-renewing versus differentiating cells remained almost constant under all conditions tested (Figure 4B-C). One reason for this apparent discrepancy (see "Discussion") lies in the technical principle of EMSAs. This assay for native IRE/IRP complexes using radiolabeled IRE transcripts detects free IRP not already stably associated with endogenous unlabeled IREs.[58] Therefore, in committed erythroid cells, a higher proportion of IRPs may be associated with an increased number of IRE-bearing transcripts.

We also assessed the regulation of IRP2 expression during terminal erythropoiesis by Northern and Western blot analyses. Similar to IRP1, no significant increase in total IRP2 mRNA or protein levels was detected during differentiation; thus the decline in IRP1 mRNA-binding activity is apparently not compensated by an increase of IRP2 expression. More importantly, whereas pretreatment with Des in self-renewing cells was able to increase IRP2 protein abundance (and thus mRNA-binding activity as measured in EMSAs; Figure 4A), no iron-dependent response was detectable in differentiating erythroblasts (Figure 4C).

Iron overload and inhibition of heme synthesis restore up-regulation of Fer expression in differentiating mouse erythroblasts

Finally, we tried to gain some mechanistic insight into the question why high iron levels fail to induce Fer mRNA translation during normal differentiation of mouse erythroid progenitors. For this we used (1) FAC to test whether iron overload via this reagent would result in a cellular response, (2) unphysiologically high concentrations of Fe_2-Tf, and (3) the heme synthesis inhibitor SA. All 3 types of intervention led to a substantial increase in the protein level of FerL and FerH (Figure 5A), arguing against an iron-independent mechanism specifically inhibiting Fer mRNA translation. None of these treatments, not even addition of high Fe_2-Tf levels, resulted in significantly elevated rates of heme synthesis, but, as expected, was reduced by 70% on treatment with SA (Figure 5B). Judging by the amount of Fer synthesized, Fe_2-Tf endocytosis rates may reach saturation at about 4 mg/mL, 4-fold higher than the in vivo serum concentration. Apparently the endocytosis machinery for TfR1 internalization is not the limiting factor for hemoglobin production but rather the synthesis capacity for heme/hemoglobin itself. At present we cannot distinguish between 2 alternative explanations for our results. First, the reagents used may directly increase the so called "labile iron pool"[59,60] in the cytosol. This is likely to occur in the case of FAC, which might be taken up directly via non–TfR-based pathways[20-25]. Second, cytoplasmic iron levels might increase secondarily after efflux of excess iron from mitochondria, a plausible event in response to SA and high Fe_2-Tf treatment.

In either case our data strongly support the view that the IRE/IRP system in differentiating erythroid cells is sensing a "low-iron" state despite increasing cellular iron levels, but remains fully functional. Furthermore, the results obtained from inhibition of heme synthesis support the hypothesis that the cytoplasmic iron levels sensed by IRP may actually be kept low during erythropoiesis by a vectorial transport of iron into mitochondria[29,61-63], the site of iron insertion into protoporphyrin, thus bypassing the cytoplasmic "labile iron pool." As detailed (Figure 6), all our observations can be integrated into a comprehensive working model of how the flow of iron might occur during the phase of massive hemoglobinization.

5.1.5. Discussion

Here we demonstrate that during terminal differentiation of primary mouse erythroid progenitors, Fer mRNA translation is massively impaired, whereas translation of ALAS-E mRNA, presumed to be regulated coordinately with Fer mRNA via the IRE/IRP system, proceeds unimpeded. Furthermore, maturing erythroblasts express very high levels of TfR1, again independent of varying iron supply or IRP activity levels. These observations contrast the "standard" mode for regulation of intracellular iron metabolism in most other cell lineages, which includes (1) up-regulation of IRP mRNA-binding activity on iron depletion, which in turn (2) increases Fe_2-Tf import via stabilization of TfR1 mRNA and (3) represses iron storage via translational inhibition of Fer mRNA. Interestingly, this "standard" mode also applies to committed, self-renewing mouse erythroid progenitors, which do not yet accumulate hemoglobin and are thus independent of mechanisms to ensure high iron uptake. In contrast, terminal differentiation into erythrocytes uncouples the coordinate regulation of Fer and ALAS-E mRNA translation, and elevated expression of TfR1 persists despite the presence of high (physiologic) levels of Fe_2-Tf. This type of regulation is perfectly suited to ensure maximum hemoglobin accumulation but difficult to reconcile mechanistically with the "standard" model.

Previously we reported related findings for committed or differentiating chicken erythroid progenitors[55]. There, (1) TfR1 levels were very high, even under saturating doses of Fe_2-Tf, involving transcriptional and posttranscriptional mechanisms[15,25,55,64,65]; (2) Fer mRNA translation was massively impaired and could not be modulated by iron[40], whereas (3) ALAS-E mRNA was translated efficiently[40]. Nevertheless, avian erythroblasts differ in several aspects from those in mammals, for example, in their lack of enucleation. Furthermore, apparently missing expression of FerL[40] and deviation in the hexa-loop consensus sequence (5'-CAGUGN-3' 5'-CAGCGN-3') of the ALAS-E-IRE[40] could result in differences of iron metabolism during avian versus mammalian erythropoiesis.

Obviously, based on both, chicken and mouse data, the regulation model of iron metabolism needed extension to account for specific requirements of maturing erythroblasts, which have to establish exceedingly high rates of iron uptake for successful hemoglobinization without activating iron storage. We addressed this problem using primary mouse erythroblasts[32,33], which expand more than 10^7-fold in serum-free media and undergo terminal differentiation in response to Epo plus insulin (Figure 1). In

polysome gradient analyses, we compared iron-dependent translational regulation of FerH, FerL, and ALAS-E mRNAs. "Low" iron conditions were simulated by addition of the iron chelator Des, "high" iron conditions were achieved by supplementing the medium with physiologic levels of Fe_2-Tf (1 mg/mL). In immature, self-renewing erythroblasts, translation of all 3 transcripts was still coordinately regulated by the availability of iron. During advanced stages of erythroid maturation, however, translation of both Fer transcripts was almost completely blocked, irrespective of iron (Figure 2A-B). In contrast, the fraction of polysome-bound ALAS-E mRNA almost doubled during differentiation. Furthermore, there was a more than 10-fold increase in total ALAS-E mRNA (Figure 2D). Interestingly, the 2.5-fold difference in the amount of polysome-bound ALAS-E transcripts (2.4-fold in protein expression) between samples treated with or without iron chelator only slightly affected hemoglobin formation (15% ± 2%; not shown). This suggests that ALAS-E alone is not limiting for hemoglobinization under physiologic conditions (12.5 μMFe_2-Tf), in line with our observation that in ALA assays the factor of regulation by iron was smaller (1.6-fold ± 0.1) than on the level of ALAS-E-protein or -mRNA. These and related findings by others are most likely due to compensatory mechanisms[66,67]. A potential contribution of the non–IRE-containing isoform of ALAS[68] is unlikely, because ALAS-$E^{-/-}$ mouse embryos have no hemoglobinized cells and die at day E11.5, at the onset of fetal liver erythropoiesis[26]

Why then should ALAS-E mRNA expression be regulated by iron at all? Translational repression in self-renewing cells, together with other mechanisms, might help to avoid premature onset of heme synthesis. One might further assume that, together with high expression of TfR1 mRNA, massive transcriptional activation of the *ALAS-E* gene leads to an excess of IRE sites over available IRP molecules. Thus abundant de novo synthesized ALAS-E transcripts would escape this inhibitory interaction due to the limiting amount of "free" IRPs available.

Reduced availability of IRPs and increase in IREs may be, however, insufficient to fully explain the observed uncoupling of translation efficiency between Fer and ALAS-E mRNAs during erythropoiesis. A second important factor in the translational activation of ALAS-E mRNA may be the potentially different role of IRP1 versus IRP2 in erythropoiesis. As recently reported, IRP2 knockout mice exhibit microcytic anemia[69,70]. Although there are discrepancies regarding the regulation of Fer and ALAS-E in erythroid cells from these animals, which may arise from the use of total bone marrow cells versus sorted erythroid progenitors, both reports describe down-regulation of TfR1 in the erythroid compartment. On the other hand, mice lacking IRP1 did not show an erythroid phenotype. Thus, in mice, IRP2 can compensate for the loss of IRP1 but not vice versa[71].

This, however, does not rule out an important function for IRP1 during erythropoiesis because heme synthesis in zebrafish is strongly connected to regulation of IRP1 binding activity[72]. Moreover, the microcytic anemia observed in IRP2$^{-/-}$ mice is more severe in an IRP1$^{+/-}$ background, again arguing for a contribution of IRP1 to iron regulation in erythroid cells. These in vivo data and in vitro experiments showing a higher affinity of IRP2 for bulge/loop hairpins as in Fer mRNA[73,74] plus high affinity of IRP2 for the multiple IREs in the context of TfR mRNA[75] could explain the difference in IRE-mediated translational inhibition via IRPs in erythroid cells; IRP2 might be mainly responsible for regulation of TfR1, whereas IRP1 would preferentially modulate expression of ALAS-E. At present this scenario is speculative, especially because the existing knockout data on the roles of IRPs in the hematopoietic lineage are somewhat contradictory.

The observations described in this paper would fit to the so called "kiss-and-run" hypothesis[29,62]. It suggests that during terminal erythropoiesis endosomes come into close vicinity/physical contact with mitochondria to directly shuttle iron into this organelle for heme synthesis[61,63]. Thus, intermediary release of iron from endosomes into the cytosol would be avoided, rendering the metal concentration "low" for the IRE/IRP system. Alternatively, and to the same consequence, increased activity of mitochondrial iron importers may prevent metal accumulation in the cytosol. Increased iron influx into mitochondria also might explain why ALAS-E but not TfR1 or Fer mRNA remains subject to translational control during erythroid differentiation. Excess iron not incorporated into heme can be used for Fe-S cluster synthesis, which on export from mitochondria will reduce IRP1 mRNA-binding activity. Consequently, as described, this might activate mainly ALAS-E mRNA translation, as detected in polysome gradients and immunoprecipitations.

Building on the "kiss-and-run" hypothesis, we put our data into a working model of iron utilization in late-stage erythropoiesis (Figure 6). During differentiation, ALAS-E mRNA abundance increases. Because total IRP1 and IRP2 protein levels do not rise in parallel, IRP may become limiting. This might primarily affect ALAS-E mRNA due to the lower affinity of IRP1 for its IRE[73,74], resulting in enhanced ALAS-E synthesis. The increase in TfR1 expression can be reconciled with potentially limited availability of IRPs by a decline in the activity of the endonuclease involved in TfR mRNA turnover, as in chicken[55]. At the same time, endosomes might increasingly shuttle their iron load directly toward mitochondria, requiring coordinated and directed vesicle flow (Figure 6A)[61-63], most likely involving the iron transporter DMT1[76-78]. In differentiating erythroblasts endosomes become increasingly acidified, favoring release of Fe from Fe_2-Tf and its export[56]. Furthermore, endosomes are redistributed from the cell periphery toward the

deeper intracellular space, confirmed by confocal laser and electron microscopy (Lioba Lobmayr and Iris Killisch, unpublished data, June 2004). Thus, the cytosol may again be recognized as "low iron" by the IRE/IRP system, despite a massive net increase of iron import into the cell. Within mitochondria, iron will be efficiently incorporated in protoporphyrin IX by ferrochelatase to form heme, which is assembled into hemoglobin immediately after mitochondrial export.

To test the idea of a "low iron" cytosol, we artificially increased the cytosolic iron pool. For this we either inhibited heme biosynthesis or overloaded the cells with iron (Figure 6B). A block of heme synthesis by SA, an inhibitor of ALA-D[62], resulted in a significant increase in Fer protein. Most likely, this was due to an efflux of non–heme-bound iron via the mitochondrial iron export machinery[77], inducing secondary cytosolic iron overload (as confirmed by others[26]). In turn, this should reduce IRP mRNA-binding activity and actually increased Fer protein expression. Fer protein levels were similarly up-regulated on iron overload with FAC, which enters many cell types directly, bypassing TfR-mediated endocytosis[20-23]. However, TfR-independent FAC uptake has not been rigorously confirmed for mouse erythroid progenitors so far[24,25,79].

At present, we cannot address whether or not mitochondrial Fer[80,81] may influence iron efflux versus storage in mitochondria. Although mitochondrial Fer has high homology to cytosolic Fer and is abundant in mitochondria of human patients with sideroblastic anemia, its role in healthy individuals is unclear[81,82].

Here we present a comprehensive analysis of key players regulating iron metabolism in self-renewing versus differentiating primary mouse erythroblasts. Terminal erythropoiesis caused a switch of regulation to a mode where the IRP/IRE system sensed a low-iron state despite massively increased iron uptake, intracellular transport, and utilization for hemoglobin synthesis. The altered iron metabolism in differentiating erythroblasts may have evolved to ensure maintenance of high levels of TfR1 mRNA for rapid uptake of large amounts of Fe_2-Tf. This mode of regulation should be important to ensure efficient heme production by synthesizing high levels of ALAS-E without favoring Fer mRNA translation, thus avoiding futile iron storage during a phase of high iron demand, a situation known to disturb hemoglobinization[31].

5.1.6. Acknowledgements

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5.1.7. Figures

Figure 1

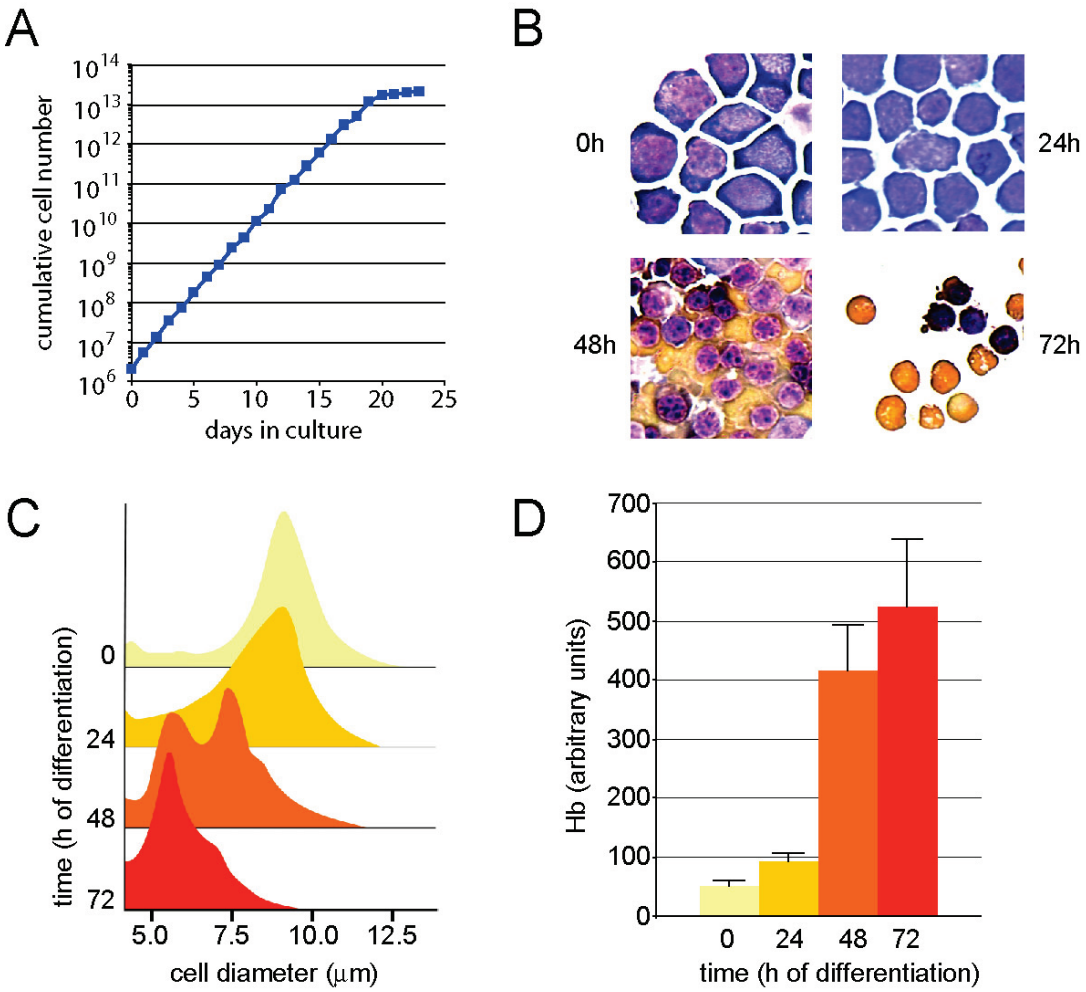
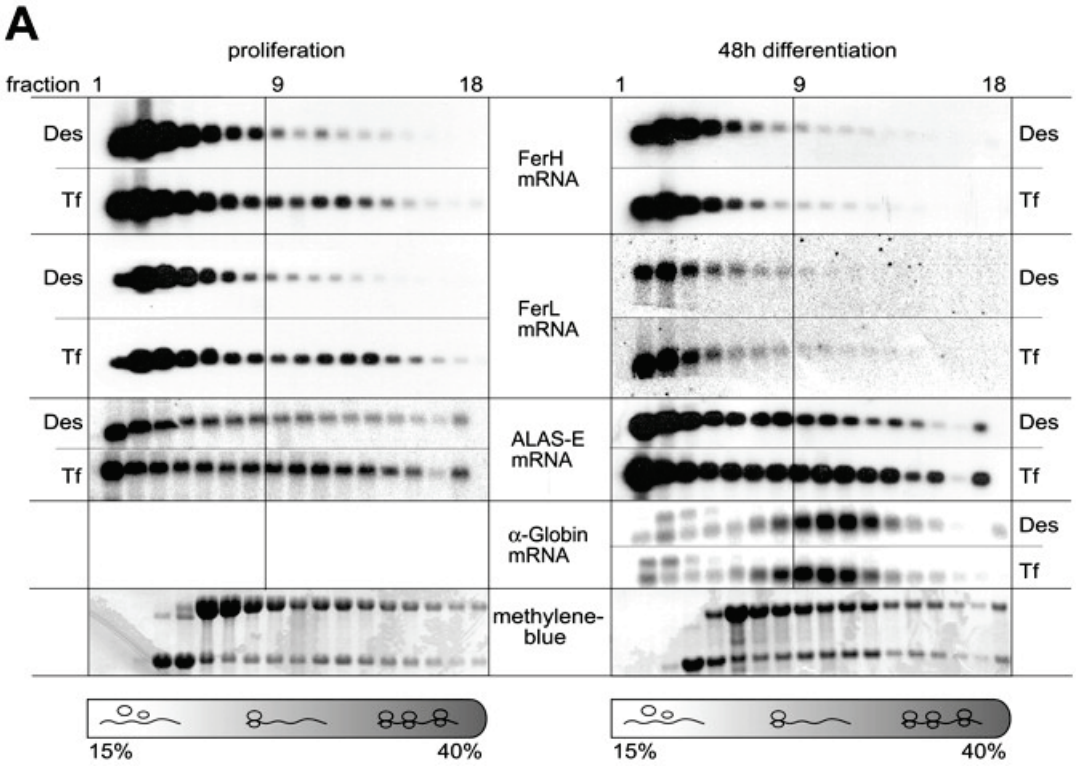
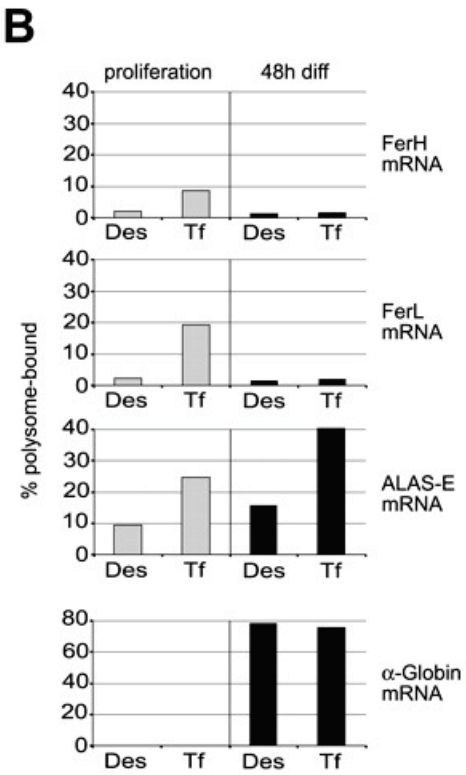


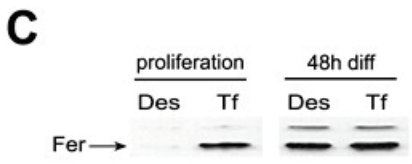
Figure 1. Extended self-renewal and synchronous differentiation of primary, fetal liver–derived mouse erythroblasts. (A) Cells from fetal livers of E12.5 mouse embryos were cultivated in serum-free StemPro (Life Technologies) medium plus NutriMix supplement in the presence of stem cell factor (SCF), erythropoietin (Epo; 2 U/mL), and the synthetic glucocorticoid dexamethasone (Dex). Proliferation kinetics of outgrowing erythroblasts were determined by daily measurements of aliquots in an electronic cell counter (CASY) and cumulative cell numbers calculated as described[50]. (B) Terminal differentiation was induced by replacing proliferation factors with insulin, the glucocorticoid antagonist ZK112993[34] plus high levels of Epo (10 U/mL) and Fe_2-Tf (1 mg/mL = 12.5 µM). To monitor morphologic changes in maturing cells, aliquots were withdrawn at daily intervals, cytocentrifuged onto slides, and stained with neutral benzidine (to detect hemoglobin; brownish stain) and histologic dyes[36]. Note size

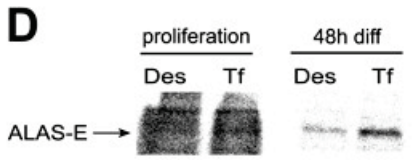
decrease and enucleation of mature cells (72 hours, bottom right panel). Photomicrographs were taken using an Axiovert 10 microscope (Zeiss, Oberkochen, Germany) equipped with a 63 x oil-immersion objective lens (numerical aperture 44-07-61; Zeiss). Images are presented at original magnification, x 630. Images were captured with a Sony 3CCD color video camera (Sony, Tokyo, Japan) and prepared for publication with IP Lab Spectrum P software 3.1.1 (Signal Analytics, Vienna, VA). (C) Measurements of the decline in cell volume during differentiation were performed with an electronic multichannel cell analyzer. Appearance of 5-µm peak indicates mature cells with volumes close to that of peripheral blood erythrocytes. (D) Hemoglobin levels during differentiation were quantitated using a photometric assay previously described, and normalized to both cell numbers and cell volume from 50-µL aliquots in triplicate[32,37]; error bars, SD of mean, n = 4.

Figure 2


A
proliferation
48h differentiation
fraction 1
9
18
Des
Tf
FerH mRNA
FerL mRNA
ALAS-E mRNA
α-Globin mRNA
methylene-blue
15%
40%


B
proliferation
48h diff
% polysome-bound
FerH mRNA
FerL mRNA
ALAS-E mRNA
α-Globin mRNA
Des
Tf


C
proliferation
48h diff
Des
Tf
Fer


D
proliferation
48h diff
Des
Tf
ALAS-E

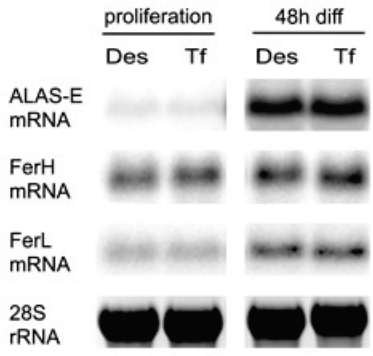

E
proliferation
48h diff
Des
Tf
ALAS-E mRNA
FerH mRNA
FerL mRNA
28S rRNA

Figure 2. Translational repression of Fer mRNA and efficient utilization of ALAS-E mRNA in differentiating mouse erythroblasts. Self-renewing (designated "proliferation" in this and the following panels) or differentiating (labeled "48h diff") primary mouse erythroblasts were incubated with the iron chelator desferrioxamine (Des, 50 μM) or physiologic concentrations of iron-loaded human transferrin (Tf, 12.5 μM) for 24 hours prior to harvesting. (A) Polysome gradient analysis. Cytoplasmic extracts were separated in linear 15% to 40% sucrose gradients[39] and the RNA isolated from 18 fractions analyzed by Northern blotting. Fraction 1, top, fraction 18, bottom of the gradient. Filters were sequentially hybridized with [^{32}P]-labeled probes specific for mouse FerH, FerL, ALAS-E, and (in the case of differentiating cells) α-globin mRNA as control. Bottom panel, loading control; methylene blue stain of total RNA. The constant molar ratio between 28S and 18S RNA (top and bottom band, respectively) around fraction 9 indicates the assembly of 80S initiation complexes and marks the approximate boundary between the ribosome-free, untranslated, and polyribosome-bound, translated mRNA compartment, as schematically depicted at the bottom. (B) Quantification of polysome-bound, translated mRNA. Bar diagrams depict the sum of the percentage of mRNA in fractions 9-18 as determined by PhosphoImage analysis. (C) Fer protein expression in proliferating and differentiating cells. The antibody used (see "Materials and methods") recognizes both FerH and FerL. (D) ALAS-E expression as determined by immunoprecipitation of cell extracts (normalized to equal number of counts per sample) pulse labeled for 20 minutes with [^{35}S]-methionine; to visualize the ALAS-E band in proliferating cells, this signal was amplified electronically 5 times. (E) Total mRNA levels for ALAS-E, FerH, and FerL mRNAs. Loading and quality control, 28S rRNA stained with methylene blue.

Figure 3

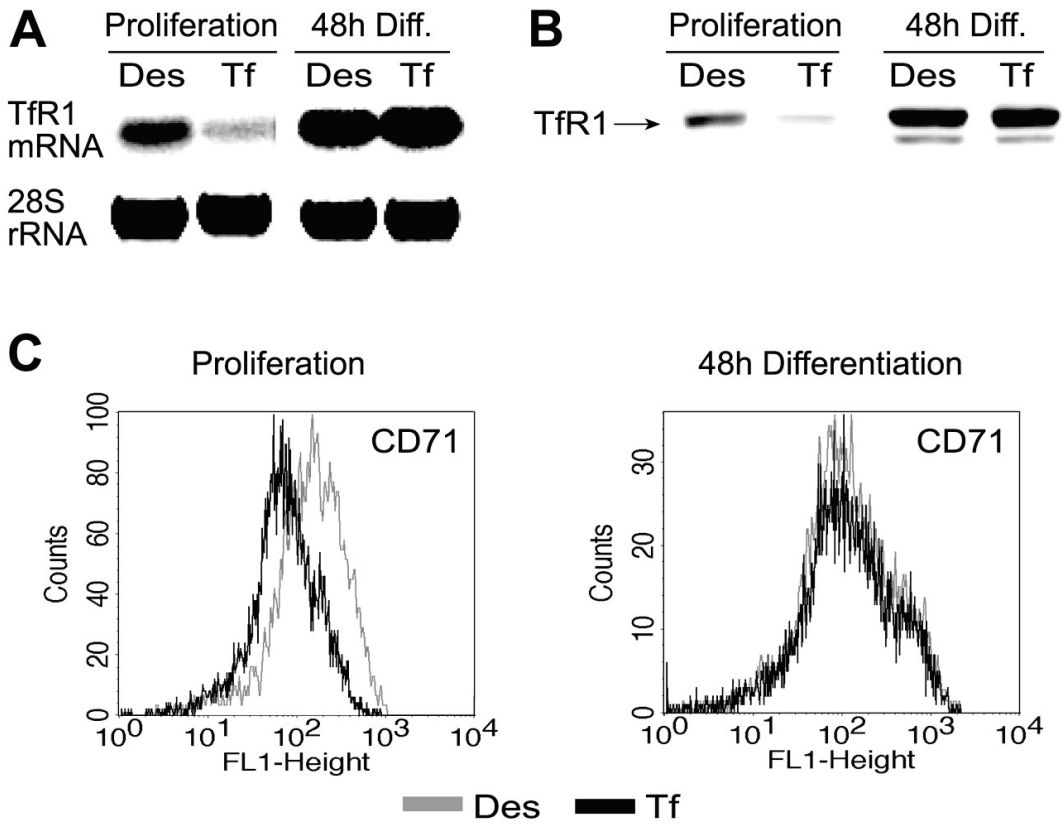


Figure 3. Transferrin receptor expression is independent of iron in differentiating mouse erythroblasts. Fetal liver–derived mouse erythroid progenitors pretreated as described in the legend to Figure 2 were analyzed for transferrin receptor (TfR1) expression. (A) TfR1 mRNA determined by Northern blotting (28S rRNA hybridization as control); (B) total TfR1 protein determined by Western blotting; Erk1/2, loading control. (C) TfR1 cell-surface expression was determined by flow cytometry.

Figure 4

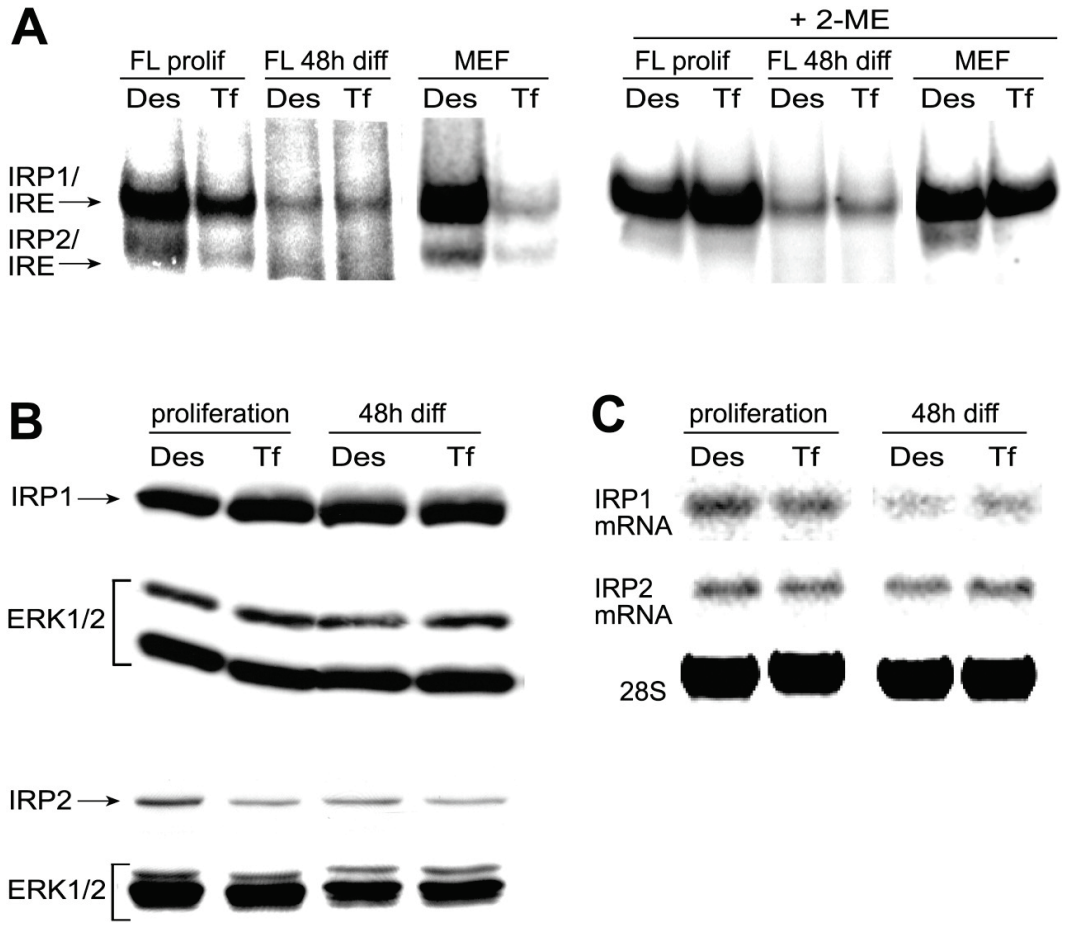


Figure 4. mRNA-binding activity of IRP is independent of iron in differentiating primary erythroblasts. (A) Determination of apparent (left panels) and total IRP mRNA-binding activities (right panels; +2-ME[43]) in extracts of mouse erythroblasts (designated "FL," for fetal liver–derived cells), pretreated as described in Figures 2 and 3. Electrophoretic mobility shift assays (EMSAs) of complexes between IRP and radiolabeled in vitro–transcribed RNAs containing the IREs of mouse FerH mRNA (clone 42)[42] were performed as described in "Materials and methods." Control extracts, demonstrating the full regulatory potential of IRP, were prepared from mouse embryo fibroblasts (MEFs). Total IRP1 and IRP2 protein (B) and mRNA levels (C) were determined by Western (Erk1/2 used as loading control) and Northern blotting (28S rRNA signal as RNA quality and loading control), respectively.

Figure 5

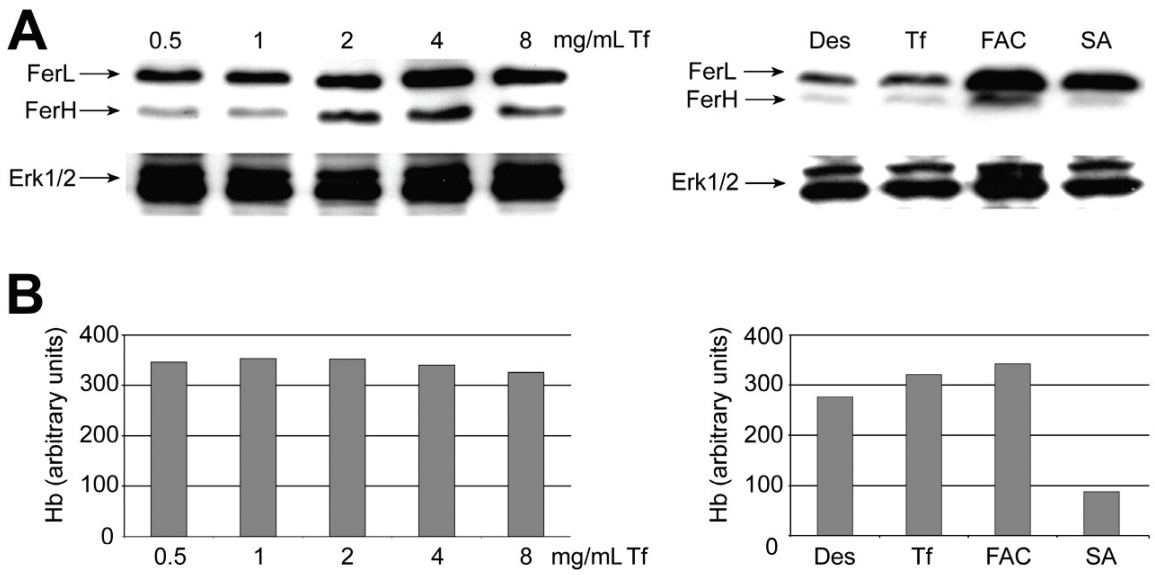


Figure 5. Iron overload and inhibition of heme synthesis restore iron-dependent Fer expression in differentiating mouse erythroblasts. Fer expression as determined by Western blotting in erythroid progenitors differentiating for 48 hours. (A) Cells were either incubated for 24 hours with Fe_2-Tf (6.3-100 µM; highest concentration corresponds to 8 times the physiologic level; top left panel), or Des (50 µM), Fe_2-Tf (12.5 µM), FAC (63 µM Fe) and SA (0.2 µM; inhibition of heme synthesis) (top right panel). FAC probably can enter the cells directly, bypassing Tf/TfR-mediated endocytosis and the assumed vectorial iron transport from endosomes into mitochondria, and may thus lead to direct cytosolic iron overload ERK1/2 (bottom panels), loading control; membranes restained with corresponding antibody. (B) Hemoglobin synthesis in the cells described in panel A.

Figure 6

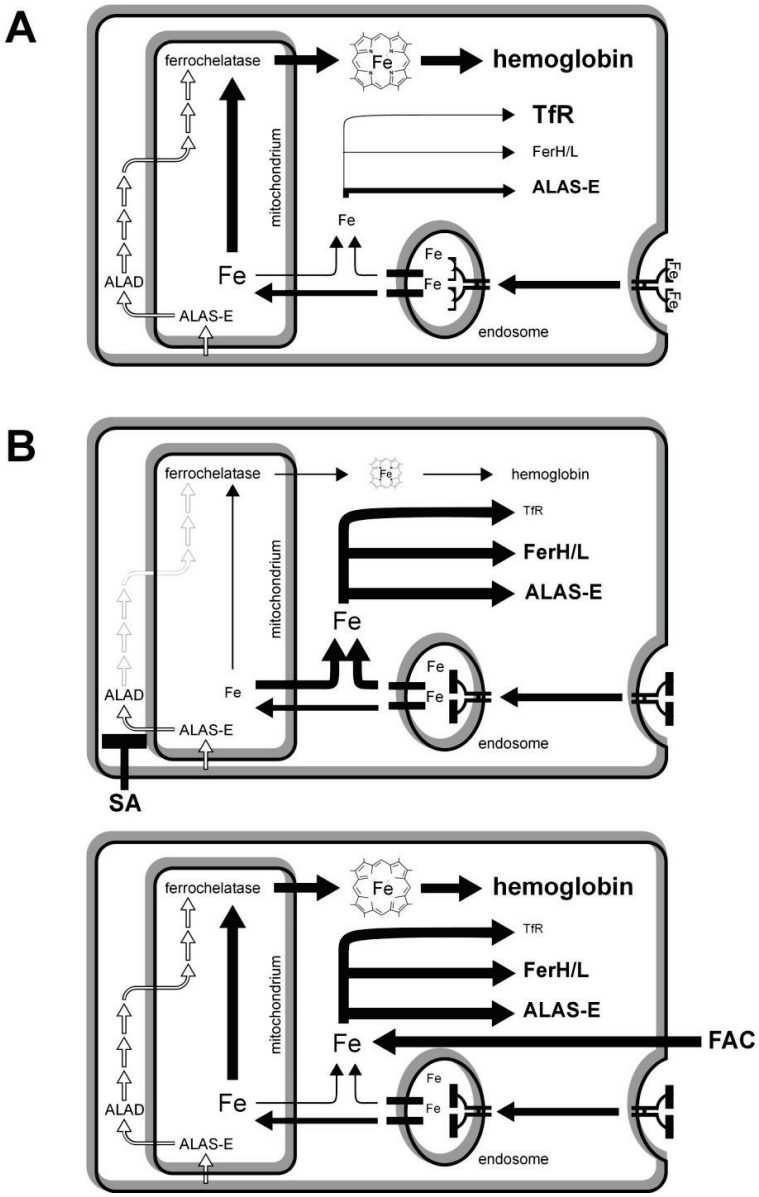


Figure 6. Working model for the regulation of iron metabolism in differentiating primary erythroblasts. (A) The model shown in this scheme is essentially based on the "kiss-and-run" hypothesis[62] of vectorial iron transport toward mitochondria. It depicts the distribution of iron (cytosolic versus mitochondrial) in differentiating erythroid cells as well as how the expression levels of TfR1, Fer, and ALAS-E are regulated via IRP. Thick and thin black arrows symbolize high and low rates of iron flow, respectively; open white arrows depict heme synthesis; lettering size for hemoglobin, TfR1, Fer, ALAS-E and "Fe" (iron-loaded heme) indicates the expression level of the corresponding protein or compound. (B) Predicted and in part experimentally verified consequences of (1) perturbation of mitochondrial iron uptake/flow by adding inhibitors of heme biosynthesis like succinylacetone (SA; upper panel) or (2) of direct cytoplasmic iron overload with low-molecular-weight iron salts (ie, addition of ferric ammonium citrate, FAC; lower panel).

5.1.7. References

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5.2. Low cytosolic non-heme iron levels in erythroid cells prevent IRP2-mediated ferritin upregulation during differentiation

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5.2.1. Abstract

Differentiating erythroid cells shuttle iron with very high efficiency towards mitochondria for the formation of heme. Recently we have demonstrated that primary erythroid cells satisfy their exceptionally high requirements for iron during terminal differentiation by switching to a mode where the post-transcriptional, iron-dependent regulatory system, based on iron responsive proteins (IRP1 and IRP2) and iron responsive elements (IREs), seems to sense low iron levels within cells. This occurs despite a massive net increase of iron import into cells[1].

To examine the hypothesis that erythroid cells have low non-heme iron levels in their cytosol, we experimentally increased the cytosolic iron pool by either inhibiting heme biosynthesis or overloading cells with iron. Both treatments resulted in a clear increase in ferritin levels. Strikingly, increases in ferritin expression upon perturbation of cellular iron homeostasis strongly correlated with the loss of IRE-binding activity of IRP2 but not IRP1. This suggests that IRP2 is the major regulator of ferritin expression in erythroid cells. To further elaborate on this observation, we cultured primary erythroblasts derived from IRP1-/- and IRP2-/- mice. In agreement with the published phenotype of microcytic hypochromic anemia, only erythroblasts lacking IRP2 exhibited a reduction in hemoglobinization and showed a significant increase in ferritin expression. Together with the observation that surface expression of transferrin receptor (TfR1) was reduced in IRP2-/- erythroblasts during self renewal but not during terminal differentiation, our results suggest that not only down-regulation of TfR1, but also up-regulation of ferritin may be a major factor causing the anemic phenotype observed in IRP2-/- mice.

5.2.2. Introduction

The major function of mature red blood cells is to supply oxygen to all body tissues using hemoglobin as a docking molecule and to remove carbon dioxide in order to deliver it to the lungs. To satisfy this requirement, each erythrocyte is packed with hemoglobin molecules. Therefore, terminally differentiating erythroid cells focus their metabolic efforts on the synthesis of globin and heme. Heme biosynthesis is a very efficient process which involves eight enzymes. The first step occurs in mitochondria and involves the condensation of succinyl CoA and glycine to form 5-aminolevulinic acid (ALA), catalyzed by ALA synthase (ALAS). The next four steps take place in the cytosol and are initiated by the ALA dehydratase (ALAD). The final three steps of the biosynthetic pathway, including the insertion of ferrous iron into protoporphyrin IX (PP IX) by the last enzyme of the pathway, ferrochelatase, occur again in mitochondria.

The need for iron as a substrate for heme biosynthesis makes differentiating erythroid cells the major consumers of circulating iron. This plasma iron is bound to transferrin (Tf) which binds to the TfR1 on the cell surface. The binding is followed by internalization of the Tf/TfR1 complex via receptor mediated endocytosis. Endosomal acidification is necessary for the release of iron from the Tf-TfR1 complex[2,3]. Thereafter Fe is reduced by the ferrireductase Steap3[4] and transported across the vesicular membrane by the divalent transporter DMT1[5,6]. Finally, iron is delivered to its place of utilization. Excess Fe is trapped in the iron storage protein ferritin. This sequestration prevents the formation of cell damaging radicals by free iron.

Coordinated control between iron uptake and storage is mainly achieved by the post-transcriptional regulation of ferritin and TfR1 synthesis. Under low cellular iron concentrations the iron regulatory protein (IRP) 1 and IRP2 can independently bind to specific RNA motifs called iron responsive elements (IREs; reviewed by Pantopoulos K[7]). One type of these stem loop structures is localized in the 5' untranslated region (UTR) of both transcripts of the ferritin subunits and multiple copies are found in the 3' UTR of the TfR1 mRNA. Binding of IRPs to IREs leads to stabilization of TfR1 mRNA and consequently, to increased expression of the protein involved in iron uptake[8-10]. However, IRP/IRE complex formation in the 5' UTR of ferritin mRNA inhibits initiation of translation and prevents sequestration of iron into storage compartments[11,12]. The opposite occurs if cellular iron levels are high. Release of IRPs from IREs destabilizes TfR1 mRNA and allows for efficient synthesis of ferritin protein. Paradoxically, this would

be also the situation in terminally differentiating red blood cells which show a dramatic increase in the uptake of cellular iron needed for hemoglobinization. Therefore, according to the classical IRP/IRE sensing system, this should result not only in a down-regulation of TfR1, but also in an increase in ferritin protein expression. Hence, if this system operated in terminally differentiating RBCs iron would be diverted to iron storage instead of towards heme synthesis. In fact it has been shown that overexpression of ferritin H in a mouse erythroleukemic cell line leads to reduced hemoglobinization when these cells were induced to differentiate[13].

Recently we showed that during late stages of erythroid differentiation the regulation of key proteins in cellular iron metabolism do not correlate to cellular iron levels and are not coordinately regulated by the IRP/IRE system[1]. The view was initially that IRP1 and IRP2 may be functionally redundant; however IRP2 knock out (KO) animals exhibit a microcytic anemia[14,15], whereas IRP1 ko animals are similar to wild type (WT) animals[16]. This difference in the RBC parameter seems to be mainly due to a decreased expression of TfR1 on the cell surface of erythroid progenitors in the IRP2 knock out (KO) animals[14,15], suggesting that IRP2 has a dominant role over IRP1 in cellular iron metabolism of erythroid cells.

Using a primary erythroid cell system[17,18] we show that during differentiation efficient formation of heme keeps the cytosol in an iron-deprived state and consequently ferritin protein levels low. This translational repression is primarily achieved by IRP2. Furthermore we demonstrate that erythroid progenitors derived from IRP2 KO animals have low TfR1 expression only in the undifferentiated state but reach levels equal to WT cells during the end stage of terminal differentiation. In contrast, ferritin expression stays up-regulated during the entire differentiation process suggesting that sequestration of incoming iron by ferritin in these cells might be the major cause of the observed microcytic anemia.

5.2.3. Material and Methods

Culture of primary mouse erythroblasts

Erythroid cells were isolated and cultivated as described[17,18]. Briefly, cells were grown from fetal livers collected from E12.5 embryos (WT, MF1 background) and re-suspended in serum-free StemPro-34™ medium plus Nutrient Supplement (Invitrogen-GIBCO, Carlsbad, CA) plus 2U/mL human recombinant erythropoietin (Epo; 100ng/mL), murine recombinant stem cell factor 100ng/mL (SCF), synthetic glucocorticoid dexamethasone (Dex; 10^{-6}M) and insulin-like growth factor 1 (IGF-1; 40ng/mL). Cell number and size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany). Dead or differentiating cells were removed by Ficoll purification.

To induce terminal differentiation, continuously self-renewing erythroblasts were washed twice in PBS and seeded in StemPro-34™, containing 10U/mL Epo, insulin (Ins, $4x10^{-4}$IE/mL), Dex antagonist ZK-112993 ($3x10^{-6}$M)[19] and iron-saturated human transferrin (Fe_2-Tf; 1mg/mL=12.5µM=25µM Fe=physiological levels; Sigma, St. Louis, MO). Where indicated, heme synthesis was inhibited by 0.2mM succinylacetone (SA; Sigma)[20].

To induce iron starvation, cells were incubated with 50µM of the iron chelator desferrioxamine (Des); iron overload was induced by adding ferric ammonium citrate (FAC; 20µg/mL, 17% saturation=63µM iron) 24h before harvest.

Cell morphology, histological staining, and determination of hemoglobin content

Changes in cell morphology during differentiation were monitored by phase-contrast microscopy. For histological analysis, erythroblasts were cytocentrifuged at various stages of maturation onto glass slides and stained with histological dyes and neutral benzidine for hemoglobin as described[21]. Hemoglobin content was determined photometrically from 50µl aliquots from the cultures as described[22]. Values obtained from triplicate determinations were averaged and normalized to cell number and cell volume.

Flow cytometry

$1x10^6$ self-renewing or differentiating erythroblasts were washed twice with PBS/2% fetal calf serum (FCS) and stained with fluorescently-labeled antibodies against transferrin receptor (FITC, PharMingen, San Diego, CA, #01595). Surface marker expression was analyzed by flow cytometry (LSR-I, Becton Dickinson, Franklin Lakes, NJ) and processed with CellQuest Pro Software version 1.3.

Electrophoretic mobility shift assays

RNA-protein complexes were resolved essentially as described[23,24]. Briefly, for electrophoretic mobility shift assays (EMSAs), cytoplasmic extracts were incubated with [^{32}P]-labeled transcripts produced by T7 RNA-polymerase following linearization of the plasmid pGEM-3Zf(+)-chicken FerH-IRE with BamHI. 20µg of protein and $1.3x10^6$ dpm of labeled IRE-containing *in vitro* transcript were incubated for 20min room temperature. The total amount of IRP1 was assessed by *in vitro* reduction with 2% beta-mercaptoethanol (2-ME)[25] prior to the binding reaction. After treatment with RNAse T1 and heparin, RNA-protein complexes were resolved on 6% non-denaturing polyacrylamide gels at 4°C. Bands corresponding to IRE/IRP complexes were quantified by phospho-imaging (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis

Cell pellets were lysed in sample buffer as described[26] and 10-20µg of protein separated on SDS-polyacrylamide gels. Protein transfer and loading were visualized by staining with acidic Ponceau-S solution. Thereafter, membranes were blocked one hour at room temperature with 1% low-fat dry milk in TBS and probed overnight with anti-horse spleen ferritin (Sigma, #F-6136) or, for normalization, with anti-Erk1/2 (Sigma, #5670) or anti-eIF4E (Cell Signaling). After washing, filters were incubated with second antibody (horseradish peroxidase coupled alpha-rabbit IgG antibody (Jackson Labs., West Grove, PA, #111-035-008). After washing, immuno-reactive signals were detected by enhanced chemo-luminescence (Amersham, Buckinghamshire, England).

5.2.4. Results

Ferritin expression is iron-dependent in terminally differentiating primary erythroid cells

Although cellular iron levels increase dramatically in differentiating erythroid cells, translation of ferritin remains in a repressed state[1]. To test whether ferritin expression is still responsive to iron we employed ferric ammonium citrate (FAC) as an iron source. Both undifferentiated and differentiating (48h) erythroblasts were incubated for 24h with FAC. Western blot analysis shows that increasing concentrations of FAC correlate with increasing expression of ferritin (Figure 1). Thus, these data argue against the possibility that an iron independent mechanism specifically inhibits ferritin mRNA synthesis during terminal differentiation.

Inhibition of heme synthesis activates expression of ferritin in differentiating erythroblasts

The switch to a repressed state of ferritin levels despite a net increase in cellular iron correlates with a rise in hemoglobinization (data not shown). In order to test for a link between heme biosynthesis and regulation of ferritin expression, we blocked heme biosynthesis at two different stages. First, we used succinylacetone (SA) to inhibit ALAD, the second enzyme of the heme biosynthesis pathway[20]. As shown in Figure 2A, inhibition of ALAD by 0.2mM SA for 24h clearly reduced heme biosynthesis and led to an equivalent increase in ferritin protein expression (Figure 2B). This indicates that functional heme biosynthesis is necessary to keep ferritin expression at low levels.

Ferritin expression negatively correlates with heme synthesis

Secondly, we used isonicotinic hydrazide (INH) to inhibit aminolevulinic acid synthase (ALAS-E), the first enzyme of the heme synthesis pathway. As shown in Figure 3A inhibition of heme production by INH was comparable to the inhibitory effect of SA on 48h differentiated erythroblasts. INH causes a similar block of heme biosynthesis and a marked increase in ferritin protein expression (Figure 3B). The block of heme biosynthesis by INH could be partially restored by addition of ALA. As shown in Figure 3,

0.5mM and 1mM ALA can re-establish about 30% and 60% of heme biosynthesis, respectively. Strikingly, with augmented restoration of heme formation we obtain a parallel decrease in ferritin protein levels as shown by western blot analysis. Thus, this data supports the view of an inverse correlation between heme biosynthesis and ferritin protein expression.

Cytosolic iron overload by FAC or inhibition of heme synthesis is sensed by IRP2 but not IRP1

To determine if the incubation with FAC or the inhibition of heme synthesis that were both followed by an upregulation of ferritin changed the binding activities of IRPs we performed electro mobility shift assay (EMSA) using a radio-labeled IRE probe and looked at complex formation between this probe and IRP1 and IRP2. As previously shown, incubation of both undifferentiated and differentiated erythroid cells with FAC increased the expression of ferritin in those cells (Figure 4A, left panel). Again the same is true upon treatment of 48h differentiated erythroblasts by SA only or in combination with FAC (Figure 4, right panel). Following analysis of IRP1 and IRP2 binding activity, however, only the formation of IRP2/IRE complexes was reduced, whereas the binding activity of IRP1 is unaffected (Figure 4B). Because decrease in binding activity of IRP2 nicely correlates with the increase in ferritin expression, we conclude that changes in the cellular iron balance by administration of FAC or inhibition of heme synthesis by SA are mainly sensed by IRP2 and not by IRP1. Therefore we postulate that translational regulation of ferritin in these cells is primarily achieved by IRP2 and not by IRP1.

IRP2 is the major regulator of iron-dependent expression of ferritin

To further investigate the possibility that the translation of ferritin is mainly regulated by IRP2 and not IRP1 in erythroid cells, we used erythroid progenitors derived from fetal livers of mice deficient in IRP1 or IRP2[15]. We kept the isolated erythroblasts under either undifferentiated conditions or induced them to differentiate for 48h[17]. Expression of TfR1 was determined by fluorescence-activated cell sorting analysis (FACS). In agreement with *in vivo* data[14,15] only undifferentiated erythroblasts deficient in IRP2 but not those deficient in IRP1 showed a reduced expression of TfR1 on their cell surface (Figure 5C). Moreover only erythroid cells derived from IRP2 KO animals showed a decrease in hemoglobin accumulation after 48h of differentiation (Figure 5A), in agreement with the

microcytic anemia observed only in these animals[14,15]. Western blot analysis of ferritin expression in undifferentiated and 48h differentiated erythroblasts demonstrated that ferritin protein was strongly elevated in IRP2-/- cells, but not in IRP1-/- cells as compared to the WT counterpart (Figure 5B). These results underline the notion that IRP2 is the major translational regulator of ferritin expression in erythroid cells.

Moreover, the TfR1 expression on the cell surface was compared between undifferentiated erythroblast derived from KO and WT animals and their differentiated counterparts (Figure 5C). We could not detect any significant difference in the cell surface expression of TfR1 during the late stage of erythroid differentiation between all three genotypes (Figure 5C, right panel). Hence TfR1 is downregulated in IRP2 deficient erythroblasts at a stage of low hemoglobinization, whereas ferritin expression is upregulated throughout the whole terminal differentiation process. This suggests that the microcytic anemia observed in IRP2 KO animals might be less a result from downregulation of TfR1 during early stages of terminal erythropoiesis but primarily a consequence of up-regulated ferritin expression during the phase of high hemoglobinization.

5.2.5. Discussion

Erythroid cells are the major consumers of iron in the human body. Differentiating erythroid cells shuttle the metal with very high efficiency towards mitochondria in order to use it for the formation of heme[27]. To satisfy their high iron needs, developing red blood cells (RBC) have to sustain high expression of TfR1 despite increasing cellular iron concentration. Moreover, synthesis of ferritin must not be activated by incoming iron, since this would represent a counterproductive storage during the phase of high iron demand. Recently we have demonstrated that during terminal differentiation primary erythroid cells maintain high TfR1 expression and low ferritin expression despite a massive net increase of iron import into the cell[1].

We have hypothesized that erythroid cells have low non-heme iron levels in their cytosol and show in this report that both block of heme synthesis by either SA or INH or administration of FAC, resulted in a clear increase in ferritin levels. Moreover, the effect of INH, the inhibitor of ALAS2, could be reversed by the addition of ALA.

The positive correlation between increasing concentrations of FAC treatment and the increased expression of ferritin protein demonstrates that ferritin expression is still responsive to iron in differentiating erythroblasts (Figure 1). This eliminates the possibility of an iron-independent inhibition of the translational regulation of ferritin. Similarly the inhibition of heme synthesis increases the ferritin expression, however it remains to be elucidated how this treatments increase the regulatory iron pool. Basically, there are two possibilities upon treatment with FAC or inhibitors of heme synthesis: (I) once iron is released from endosomes it accumulates in the cytosol and this regulatory iron pool inactivates binding activity of IRP2[7]; (II) iron is shuttled directly into mitochondria[28] and is then released from this organelle when the iron availability exceeds the iron demand and so increases the regulatory iron pool in the cytosol. The data presented here cannot answer this question and we are currently trying to address these issues.

Although it is unclear how the regulatory iron pool forms upon treatment with FAC or inhibitors of heme synthesis, both treatments lead to a decreased binding activity of IRP2, but not IRP1. Therefore we suggest that expression of ferritin is mainly regulated by IRP2 in undifferentiated and differentiating erythroid cells.

This correlates with recent data using an siRNA approach showing that knockdown of IRP1 did not affect ferritin H expression, whereas knockdown of IRP2

caused an increase in ferritin expression[29]. This seems to be in contrast to *in vitro* translation assays demonstrating that IRP2 inhibited ferritin mRNA translation with a molar efficiency equal to that of IRP1 *in vitro*[30]. But additional cellular factors might tune the binding activity of the two IRPs differently *in vivo* in a tissue specific manner[31]. The view of IRP2 as a more potent regulator of the IRE than IRP1 is consistent with results obtained in knockout mice: IRP1 knockout mice display normal iron metabolism in most tissues, whereas IRP2 knockout mice show high ferritin and low TfR1 levels in multiple tissues[16]. A tissue specific effect of IRP2 deletion was shown for the erythroid compartment localized to the bone marrow. Only mice deficient for IRP2 but not IRP1 developed hypochromic microcytic anemia[14,15]. Results of this two independent groups have suggested that microcytic anemia in IRP2 deficient mice is caused by deficiency of heme synthesis due to a decreased iron uptake which results from a significant decrease in TfR1 expression on erythroid precursor cells. In agreement with these data we show that indeed proerythroblasts have a decreased TfR1 expression on the cell surface. However, this difference in expression vanished at the late stage of terminal differentiation, i.e. at a stage of highly efficient hemoglobinization. This suggests that iron uptake by erythroblasts from IRP2 KO mice may not be limited during this period. On the other hand, expression of ferritin is still high in IRP2 deficient erythroblasts at the late stage of differentiation, likely sequestering the iron into storage and thus impairing heme synthesis.

We conclude that the major factor responsible for hypochromic anemia in IRP2 KO mice is the hyper-expression of ferritin which diverts iron away from mitochondrial heme synthesis.

Erythroid cells express a specific erythroid form of the aminolevulinic acid synthase, ALAS2. In contrast to the housekeeping form, regulation of ALAS2 is independent of heme[32]. Furthermore it includes a functional IRE in its 5'-UTR. Although this constellation would suggest a regulation similar to that of ferritin by the IRE/IRP system, several studies have shown that ferritin mRNA is more potently regulated than ALAS2 mRNAs[1,34]. Furthermore, this could be potentially achieved by different roles of IRP1 and IRP2 in the translational regulation of ferritin and ALAS2. At present this scenario is speculative, especially since the existing knock out data on the roles of IRPs in mice and other animal models are somewhat contradictory. In fact studies in zebrafish have suggested that translation of ALAS2 mRNA is regulated by IRP1 and not IRP2[33]. However, analysis of ALAS2 expression in IRP1 and IRP2 KO mice support IRP2 to be the major regulator of ALAS2[14]. Latter observation would imply that differences in the translational regulation of ferritin and ALAS2 are due to differences in the structure of

their IRE leading to different binding affinities for IRP2. On the other hand Galy et al. do not observe any differences in the expression of ALAS2 between WT and KO animals. Therefore future studies on the expression of ALAS2 using erythroid cells derived from IRP1 and IRP2 KO animals should help to clarify these discrepancies.

Although in this report we mainly focused on the expression of ferritin, one very interesting observation was the normalization of the TfR1 expression at the late stage of differentiation in the IRP2 KO cells. This suggests that at this point of erythropoiesis TfR1 expression is regulated mainly at the level of transcription but not translation. Moreover it weakens the hypothesis that the microcytic anemia developed in the IRP2 KO animals is a consequence of decreased cellular iron uptake by erythroid cells.

5.2.6. Figures

Figure 1

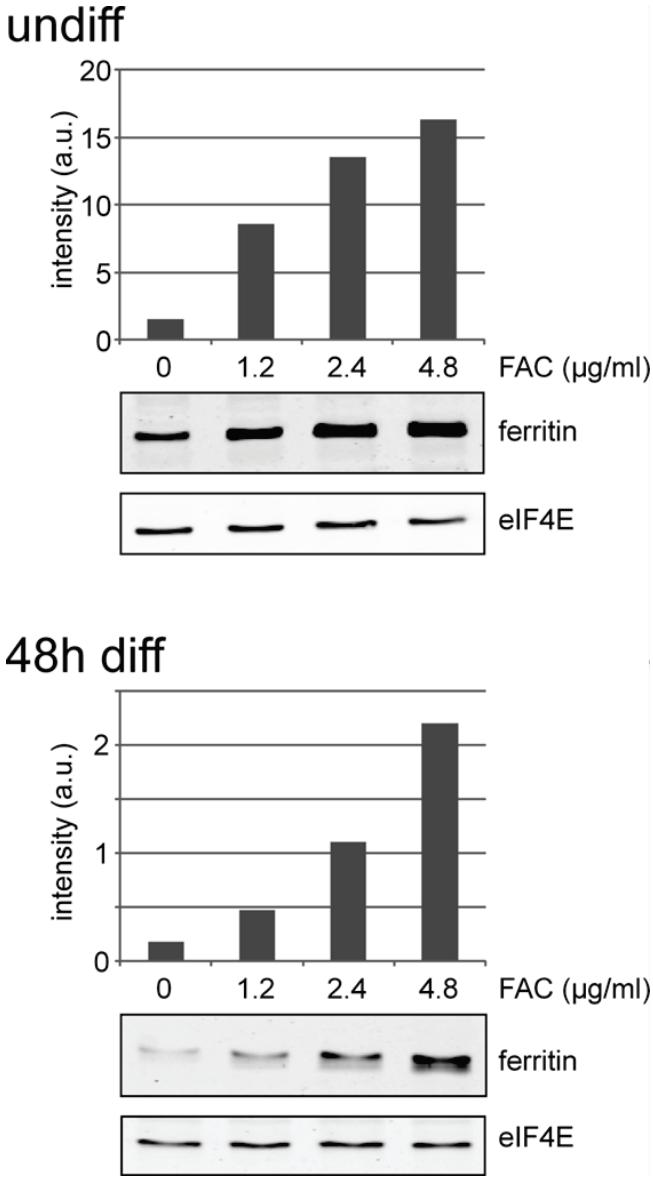

Figure 1. Ferric ammonium citrate (FAC) activates ferritin expression in undifferentiated and differentiated erythroblasts.
Undifferentiated (designated “undiff” in this and the following panels) or cells differentiating (labeled “48h diff” in this and following panels) primary mouse erythroblasts were incubated with increasing concentrations of FAC for 24 hours prior to harvesting. Bar diagrams depict the quantification by Li-Cor’s Odyssey infrared imaging system of the respective ferritin signals below obtained by western blot analysis.

Figure 2

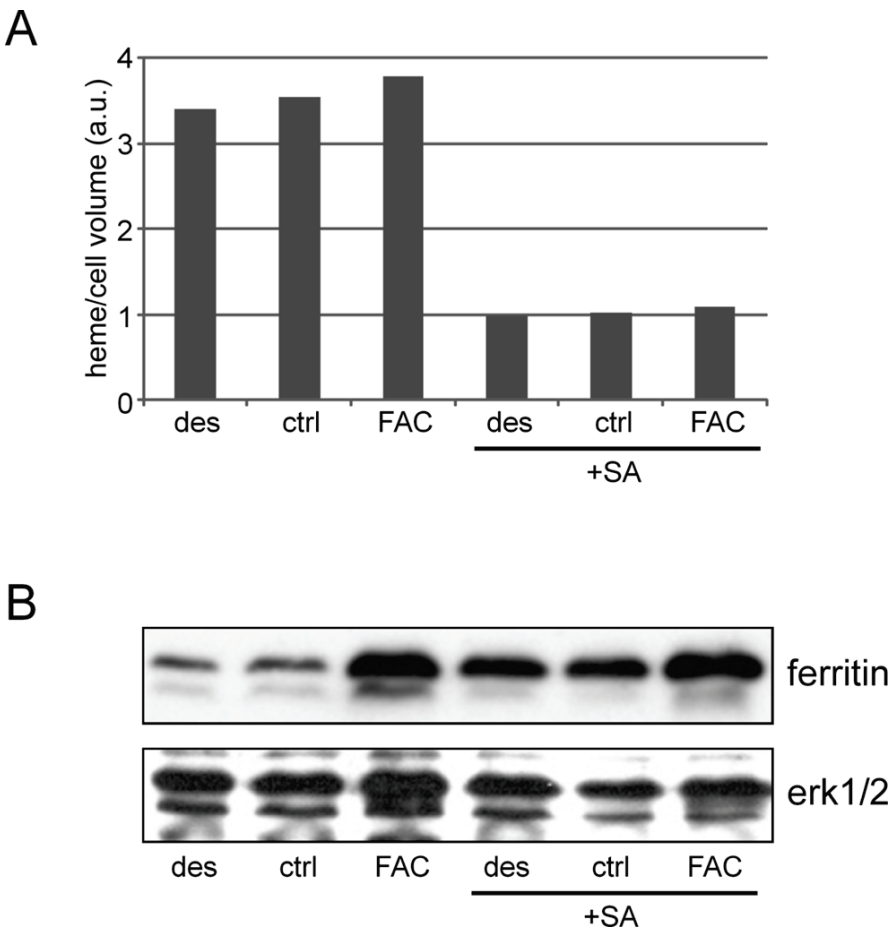

Figure 2. Inhibition of heme biosynthesis activates ferritin expression in differentiating mouse erythroblasts.

Differentiating mouse erythroblasts were incubated for 24 hours with either des (50µM) or FAC (63µM Fe) in contrast to untreated control cells (ctrl), with or without additional SA treatment (0.2mM; inhibition of heme synthesis). (A) Heme assay of the described cells. (B) Ferritin expression was determined by western blotting; Erk1/2 (lower panels) was used as loading control; membranes re-stained with corresponding antibody.

Figure 3

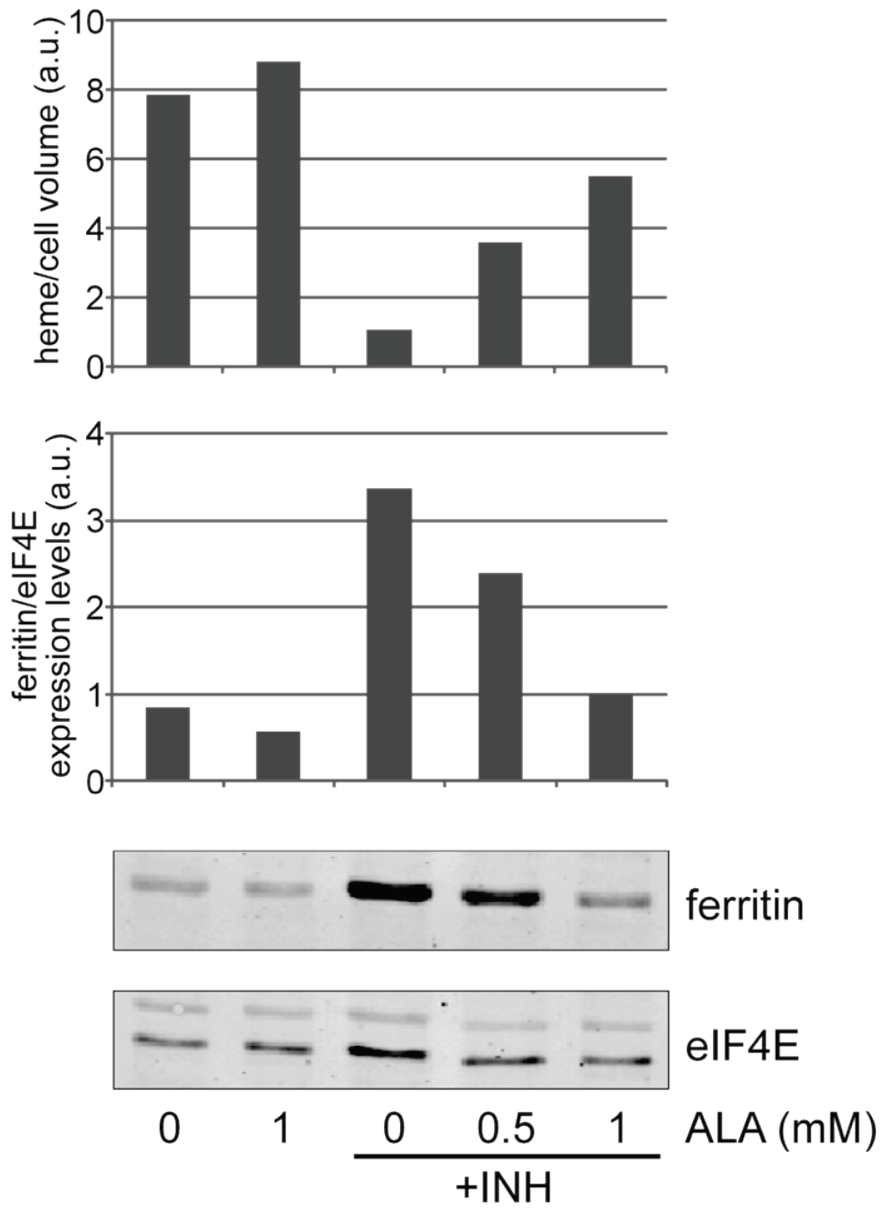

Figure 3. Expression of ferritin correlates positively with heme biosynthesis
(A) Heme biosynthesis in 48h differentiating erythroblasts was inhibited by incubation with 4mM or 8mM of INH for 24h prior to harvesting. Where indicated, cells were additionally treated with increasing concentrations of ALA to partially restore heme biosynthesis (upper panel). The heme assay was performed as described under "materials and methods". (B) Ferritin protein expression was determined by western blot analysis and quantified by Li-Cor's Odyssey infrared imaging system (bar diagrams); eIF4E (lowest panel) was used as loading control.

Figure 4

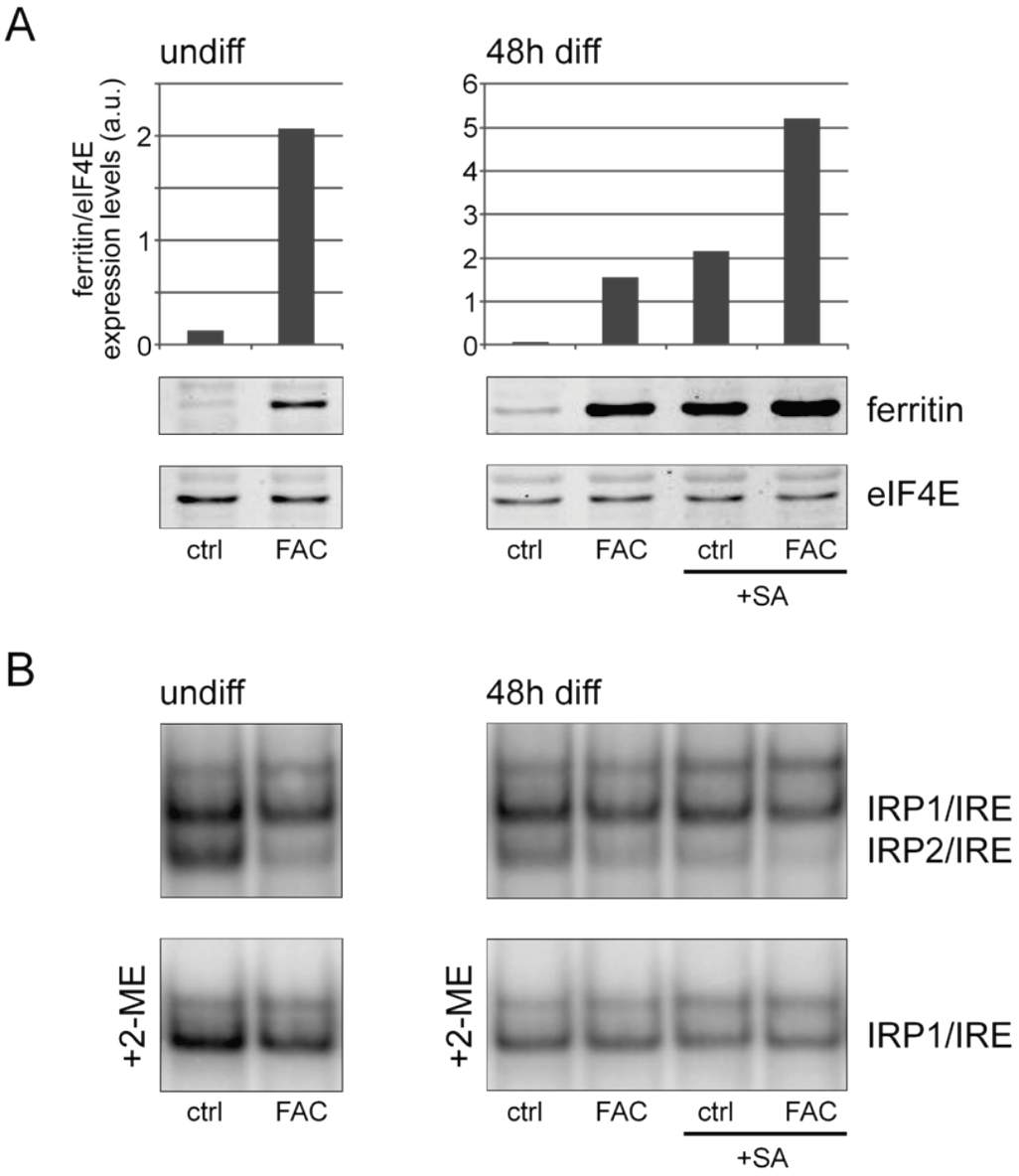

Figure 4. Binding activity of IRP2 but not IRP1 is decreased upon treatment with FAC or SA

The cell extracts of undifferentiated and 48h differentiating erythroblasts were used to determine ferritin expression and IRP binding activity. The cells were treated with FAC and/or SA as indicated (A) Western blot analysis of ferritin protein expression, eIF4E was used as loading control. (B) Aliquots of the cell extracts used for western blot were used for a band shift assay in order to determine the binding activity of IRP1 and IRP2. The total abundance of free IRP1 was monitored by the activation of non-binding IRP1 molecules by reduction with 2-mercaptoethanol (2-ME).

Figure 5

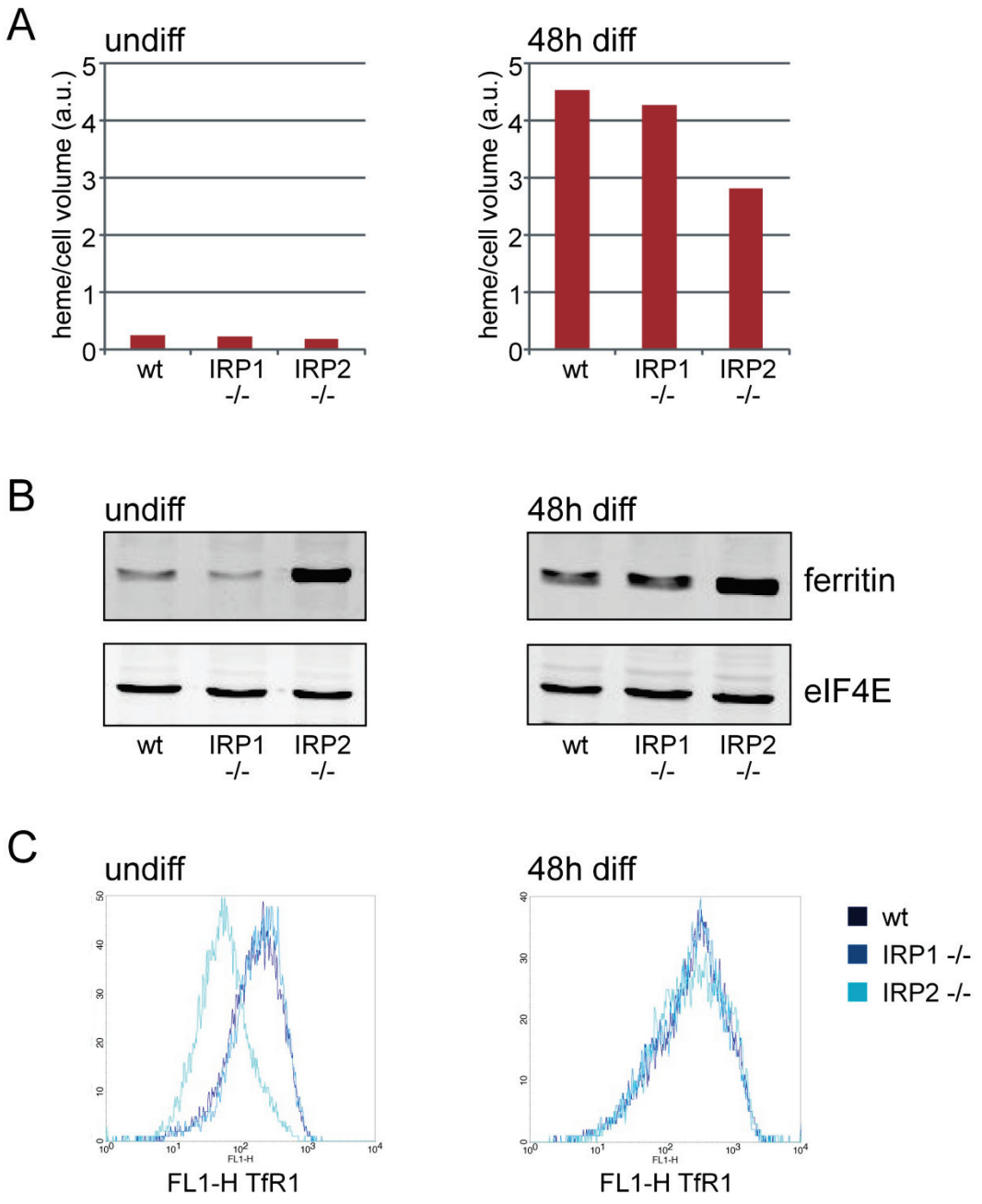

Figure 5. Analysis of hemoglobin levels, ferritin expression and TfR1 expression in erythroid cells derived from IRP KO and WT animals

Erythroblasts were isolated from fetal liver of KO and wt mice and either kept in an undifferentiated state or induced to differentiate for 48h. (A) Hemoglobin levels in undifferentiated and differentiating erythroid cells isolated from wt, IRP1-/- and/or IRP2-/- animals. (B) Ferritin protein expression was determined by western blot analysis (C) TfR1 expression was determined by FACS analysis.

5.2.7. References

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5.3. Oxygen directly regulates erythroid differentiation by modulating the activtity of 2-oxoglutarate dependent oxygenases

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5.3.1. Abstract

The availability of oxygen regulates the production of erythropoietin (epo) and thus determines the amount of newly generated erythrocytes. Furthermore, it has been shown that oxygen directly influences hematopoiesis and additionally modulates iron metabolism, which is essential during the synthesis of heme. Hence, the adjustment of the oxygen concentration during the *in vitro* cultivation of erythroblasts is of special importance.

Using a murine erythroid *in vitro* model, we show that low oxygen levels or the addition of the HIF-α activator dimethyloxalylglycine interfered with heme synthesis and cell-size decrease and promoted prolonged proliferation of immature erythroblasts. Moreover, limited oxygen supply obstructed terminal erythroid differentiation despite high epo levels. Our data suggest that the oxygen gradient in erythrocyte-forming compartments is able to directly influence the progression of erythropoiesis and that hypoxic oxygen levels interfere with terminal erythroid differentiation via stabilization of HIF-α. Furthermore, parameters like cell density and metabolic activity contribute to the effective oxygen concentration in the microenvironment of cultivated erythroblasts. Therefore, *in vitro* oxygen levels have to be individually adjusted to the specific needs of different cell types and culture conditions in order to prevent artificial effects.

5.3.2. Introduction

Oxygen is an important factor in hematopoiesis as well as in the regulation of iron metabolism. Several publications have shown that hypoxia is able to directly regulate the cycle/quiescence balance of hematopoietic stem cells and the differentiation of progenitor cells[1-4]. Furthermore, the activity of the RNA-binding iron regulatory proteins IRP1 and IRP2 that post-transcriptionally regulate the expression of several proteins involved in iron uptake, storage and utilization is not only dependent on the availability of iron but also of oxygen[5-7]. During erythropoiesis these two regulatory functions of oxygen are of special importance. It has been shown that oxygen directly affects erythropoiesis but the underlying mechanisms are not well understood [8]. In addition, reduction of systemic oxygen availability is responsible for the increased production of erythropoietin (epo) in the kidney, which is the main signal that promotes the production of mature erythrocytes in the hematopoietic tissues[9,10]. Furthermore, the influence of oxygen on the regulation of the IRE/IRP system is of special importance during erythropoiesis, since heme synthesis is dependent on the availability of iron.

Several of these aspects are analyzed *in vitro* using tissue culture incubators that use atmospheric oxygen supply. Since the oxygen levels *in vivo* are generally lower than the atmospheric concentration of 21%, the oxygen concentration has to be adjusted to physiological levels *in vitro*, when oxygen dependent mechanisms are analyzed. Although this sounds trivial, there are two major aspects that make this adjustment less straightforward: First, the oxygen concentration is not equally distributed in the body but varies in the range of 14% in arterial blood to virtually 0% in ischemic tissues[11]. The adjustment of the oxygen supply *in vitro* should therefore correspond to the correct *in vivo* concentration of the specific area of the tissue where the cultivated cell type would normally be found. Furthermore, in the special case of hematopoiesis the developing cells are confronted with increasing oxygen concentrations during maturation, thereby literally travelling throw increasingly oxygenated tissue[12]. This variation of the oxygen concentration would therefore also be necessary during *in vitro* cultivation of differentiating hematopoietic cells.

The second point that makes the adjustment of the oxygen supply comparable to the *in vivo* situation more complicated is that the modification of the oxygen concentration to a defined value within a tissue culture incubator does not necessarily mean that the cultured cells are actually facing exactly this oxygen concentration. In fact,

the oxygen supply rate in tissue culture is limited by the diffusion velocity of oxygen through the cultivation medium[13,14]. When the cultivated cells consume more oxygen than can be transferred through the medium the effective oxygen concentration becomes lower than the supplied concentration of the incubator. Therefore, additional parameters, like cell density, metabolic activity and diffusion distance also contribute to the oxygen supply of the cells and can further reduce the oxygen concentration in their direct environment[15].

We are interested in the process of erythropoiesis in general and use a murine primary cell culture system to study different aspects of this process including iron metabolism. Therefore, the influence of oxygen on this *in vitro* system is of special importance for our work. It has already been shown that the applied oxygen concentration of tissue culture incubators is able to modulate the process of erythroid differentiation *in vitro*[8]. Just recently, it has further been shown that that dimethyloxalylglycine (DMOG), an activator of hypoxia inducible factor-1α (HIF-1α) has a direct epo independent promoting influence on the proliferative capacity of the early erythroid progenitor BFU-E (burst-forming unit-erythroid)[16]. In the work presented here we used a murine primary cell culture system in order to further investigate the interplay between culture conditions, oxygen and the maturation of erythroblasts[17] . We could show that reduced oxygen levels (3%) in contrast to near-atmospheric oxygen levels (20%) can promote and extend self renewal of immature erythroblasts *in vitro*. Furthermore, the same reduced oxygen levels interfered with heme synthesis, enucleation and proliferation even in combination with factors promoting terminal erythroid differentiation. Nevertheless, we could eliminate the inhibitory effect of reduced oxygen levels by variation of cell density and diffusion distance, demonstrating their large impact on the effective oxygen supply of cultivated cells. In fact, we could show that the conventional culture conditions reduced the oxygen concentration in the direct environment of the cells compared to the oxygen concentration provided by the incubator. This demonstrates the huge impact of different tissue culture parameters that need to be evaluated in order to retrieve reproducible results especially if *in vivo* like conditions need to be established.

In addition we could show that the oxygen concentration is a potent direct regulator of erythroid differentiation and this regulation is mediated by the activity of 2-oxoglutarate-dependent oxygenases. Finally, this study shows that hypoxia is able to directly promote the generation of erythrocytes by keeping erythroblasts in an immature state longer than during normal oxygen supply and at the same time allowing terminal

differentiation of more mature erythroblasts in more oxygenated areas of the hematopoietic tissue. Hence, hypoxia is able to directly promote erythropoiesis additional to the indirect regulation via epo expression in the kidney.

5.3.3. Material and Methods

Culture of primary mouse erythroblasts

Erythroid cells were isolated and cultivated as described[17]. Briefly, cells were grown from fetal livers collected from E12.5 embryos (WT, C57BL/6 background) and re-suspended in serum-free StemPro-34™ medium plus Nutrient Supplement (Invitrogen) supplemented with human recombinant erythropoietin (Epo; 2 U/mL; Erypo, Janssen-Cilag), murine recombinant stem cell factor (SCF; 100 ng/mL; R&S Systems), synthetic glucocorticoid dexamethasone (Dex; 10^{-6} M, Sigma-Aldrich) and insulin-like growth factor 1 (IGF-1; 40 ng/mL; Promega). Cell number and size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Schärfe-System). Dead or differentiating cells were removed by Ficoll purification. Cumulative cell numbers were calculated as described[18]

To induce terminal differentiation, continuously self-renewing erythroblasts were washed twice in PBS and seeded in StemPro-34™, containing 10 U/mL Epo 10 U/ml) insulin ($4x10^{-4}$ IE/mL; Actrapid HM), Dex antagonist ZK-112993 ($3x10^{-6}$ M)[19] and iron-saturated human transferrin (1 mg/mL=25 µM Fe; Sigma-Aldrich).

All cells were cultivated in an incubator supplied with 5% CO_2 or an incubator supplied with 5% CO_2 and N_2 (Kendro). The oxygen concentration in the latter was adjusted to 3%. In order to inhibit 2-oxoglutarate–dependent dioxygenases, cells were incubated with 500 µM dimethyloxalylglycine (DMOG; Cayman Chemical).

Cell morphology, histological staining, and determination of hemoglobin content

Changes in cell morphology during differentiation were monitored by phase-contrast microscopy. For histological analysis, erythroblasts were cytocentrifuged at various stages of maturation onto glass slides and stained with histological dyes and neutral benzidine for hemoglobin as described[20]. Photomicrographs were taken using an Axiovert 10 microscope (Zeiss) equipped with a 20x lens and a 63x oil-immersion lens, a Zeiss AxioCam MRc5, and Axiovision LE software. Images are presented at x200 and x630 magnification.

Hemoglobin content was determined photometrically from 50 µl aliquots from the cultures as described[21]. Values obtained from triplicate determinations were averaged and normalized to cell number and cell volume.

Flow cytometry

$1x10^6$ self-renewing or differentiating erythroblasts were washed twice with PBS/2% fetal calf serum (FCS) and stained with fluorescently-labeled antibodies (all from BD Biosciences) against transferrin receptor (biotinylated; APC-streptavidin for detection), c-Kit (FITC-conjugated) and Ter119 (PE-conjugated). Surface marker expression was analyzed by flow cytometry (FACS Calibur, BD Biosciences) and processed with FlowJo Software (Tree Star).

Electrophoretic mobility shift assays

RNA-protein complexes were resolved essentially as described[22]. Briefly, for electrophoretic mobility shift assays (EMSAs), cytoplasmic extracts were incubated with [^{32}P]-labeled transcripts produced by T7 RNA-polymerase (Promega) following linearization of the plasmid pGEM-3Zf(+)-chicken FerH-IRE with BamHI (New England Biolabs). 40 µg of protein and $1.3x10^6$ dpm of labeled IRE-containing *in vitro* transcript were incubated for 20 minutes at room temperature. The total amount of IRP1 was assessed by *in vitro* reduction with 2% β-mercaptoethanol (2-ME)[23] prior to the binding reaction. After treatment with RNAse T1, RNA-protein complexes were resolved on 6% non-denaturing polyacrylamide gels at 4°C. Bands corresponding to IRE/IRP complexes were quantified by phospho-imaging (Molecular Dynamics).

Western blot analysis

Cell pellets were lysed in sample buffer as described[24] and 10-20 µg of protein separated on SDS-polyacrylamide gels. Protein transfer and loading were visualized by staining with acidic Ponceau-S solution. Thereafter, membranes were blocked one hour at room temperature with 1% low-fat dry milk in TBS and probed overnight with anti-horse spleen ferritin (Sigma-Aldrich), anti-mouse TfR1 (AbD Serotec) and anti-mouse eIF4E (Cell Signaling). After washing, filters were incubated with second antibody (IRDye700DX or

IRDye800DX conjugated IgG [H&L]; Rockland). After washing, immuno-reactive signals were detected with an Odyssey infrared imaging system (Li-cor Biosciences).

5.3.4. Results

The binding activity of IRPs is modulated by oxygen in terminal differentiating but not in self renewing erythroblasts

The main regulator of the binding activity of IRPs is iron. Nevertheless, other factors including oxygen are known to influence the regulation of iron metabolism via these proteins[5,6]. In order to investigate the role of oxygen in the regulation of iron metabolism during the development of erythroblasts we performed electro mobility shift assays (EMSA) with the cytosolic extracts of immature, non-differentiating as well as terminally differentiating cells (Figure 1A). First, we cultivated fetal liver derived primary erythroblasts under non-differentiating conditions and compared cultivation at low oxygen concentration (3% oxygen) to cultivation at atmospheric oxygen concentration (20% oxygen). The binding activity of IRP1 and IRP2 was only slightly affected by the different oxygen concentrations. 3% oxygen faintly reduced IRP2 binding activity whereas IRP1 binding activity was not affected. In contrast to this, terminally differentiating erythroblasts that were cultivated in 3% oxygen showed a binding activity reduction of IRP1 and IRP2 compared to cells cultivated in 20% oxygen.

Since a change of IRP binding activity affects the expression of several proteins that are linked to the iron metabolism of the cell, we also determined protein levels of TfR1 and ferritin by western blot analysis. As expected, the expression of both proteins stayed constant in non-differentiating cells (data not shown) but were considerably altered in terminally differentiating erythroblasts when the oxygen concentration was reduced (Figure 1B). Corresponding to the well known posttranslational effect on the expression of these two proteins, ferritin protein levels were increased whereas TfR1 protein levels were decreased, although the effect on ferritin was stronger.

Other groups have already shown that the IRP binding activity is modulated by oxygen[7]. In order to compare the effects observed with differentiating erythroblasts to other cell types, we incubated J774 macrophages and mouse embryonic fibroblasts (MEFs) at 3% and 20% oxygen and measured the IRP binding activity by performing EMSAs. Interestingly, these two cell types showed diverse regulation of IRP binding activity in response to changed oxygen concentrations (Figure 1C). J774-macrophages cultivated at 3% oxygen reduced their IRP1 binding activity drastically compared to 20% oxygen while IRP2 binding activity increased in these cells. In contrast to this, MEFs did not show any difference in the binding activity of IRPs under those oxygen conditions.

Therefore, the effect of oxygen on the regulation of IRP binding activity in vitro was dependent on the cell type and in the special case of erythroblasts on the developmental stage of the cells.

Low oxygen levels inhibit spontaneous differentiation during the outgrowth of immature fetal liver erythroblasts

The regulation of iron metabolism via the binding activity of IRPs is of special importance during terminal erythroid differentiation since erythroblasts depend on the uptake of high amounts of iron for the biosynthesis of heme. Down-regulation of IRP binding activity as observed in our *in vitro* culture of differentiating erythroblasts at 3% oxygen leads to down-regulation of TfR1. The iron uptake via this receptor is essential for heme synthesis during erythropoiesis and thus a decrease in its expression would interfere with the accumulation of hemoglobin. Furthermore, down-regulation of IRP also reduces the accessibility of iron within the cell by up-regulating ferritin. Available cellular iron is trapped within the nano-cages that are formed by this protein, leading to the same effect as down-regulation of TfR1. Altogether this would interfere with heme synthesis and hence with normal erythroid differentiation. Tissue oxygen levels are normally in the range of 3-5%, therefore the incubation of erythroblasts at this concentration *in vitro* is favorable compared to atmospheric 20-21% oxygen that are never reached in tissues. Nevertheless, the observed decrease of iron supply that is linked to the decrease of oxygen to "physiological levels" during a phase of high iron demand contradicts this assumption. Since we used a well defined erythroid *in vitro* system for our studies, we were able to take a closer look at this seemingly paradox situation in the context of changed oxygen supply.

First, we measured the size distribution of outgrowing murine fetal liver erythroblasts cultivated at 3% or 20% oxygen (Figure 2A). The major part of E12.5 fetal livers consists of erythroblasts of different differentiation status ranging from immature self-renewing cells to mature enucleated red blood cells. Immature murine fetal liver erythroblasts have a mean diameter of 9 to 11 µm. During the process of differentiation the diameter shrinks to about 5 µm.

During the first days of cultivation at 20% oxygen, the originally heterogeneous population was enriched in immature cells due to the signals provided by SCF, dex and epo. After 5 to 6 days in culture the cell population consisted mainly of immature erythroblasts (Figure 2A, left panel). Starting with day 7, spontaneous differentiation of

this population occurred and resulted in a mixed population of mature and immature erythroblasts, although the combination of factors was not changed. This was not the case when we cultivated fetal liver cells in the same medium but at 3% oxygen. Within 7 to 8 days these cells developed to a homogenous population of immature erythroblasts. Interestingly, these cells did not show spontaneous differentiation until day 12 of cultivation. Benzidine stainings of erythroblasts at day 7 of cultivation confirmed the above described oxygen dependent differences in cell size distribution (Figure 2B). Furthermore, the frequency of cells with a more condensed nucleus and with a hemoglobin containing cytoplasm showing a tendency for spontaneous differentiation was higher when erythroblasts were cultivated at 20% compared to 3% oxygen.

In order to test if these effects are reversible we switched the erythroblasts from 20% oxygen to 3% oxygen at day 5 (Figure 2A, middle left panel). After 2 days of adaption to the low oxygen concentration these erythroblasts remained in an immature state whereas those erythroblasts kept under normoxic conditions showed increased spontaneous differentiation. When this switch took place in the opposite direction by keeping the erythroblasts first at 3% oxygen and then shifting to 20% oxygen at day 10, the cells immediately started to differentiate and after 48 hours the major part of the population consisted of small, mature erythroblasts (Figure 2A, right panel).

Oxygen concentration modulates terminal erythroid differentiation

As shown above the oxygen concentration had an obvious effect on the outgrowth of erythroid precursors. Consequently, we also wanted to investigate whether a change of oxygen concentration affects the induced terminal erythroid differentiation. We are able to induce terminal erythroid differentiation of cultured erythroblast by the withdrawal of SCF, the replacement of dex by its antagonist ZK and the increase of epo concentration from 2 U/ml to 10 U/ml. This leads to a synchronous terminal differentiation of all erythroblasts, including substantial synthesis of heme and enucleation. In view of the fact that the iron metabolism of these cells is disturbed under low oxygen conditions (Figure 1A, right panel and Figure 1B), their general response regarding differentiation would help to clarify if this modification of iron regulation is a primary effect due to the lack of oxygen or a secondary effect of disturbed terminal erythroid differentiation.

Fetal liver cells were cultivated at 3% and 20% oxygen during erythroid outgrowth. At day 7 the cells were transferred into medium containing differentiation factors while keeping the oxygen concentrations constant. Under both oxygen conditions

the erythroblasts reduced their size during differentiation (Figure 3A). Nevertheless, erythroblasts showed a delayed size reduction when kept at 3% compared to 20% oxygen. The delayed cell size decrease of erythroblasts cultivated at 3% oxygen was accompanied by reduced proliferation, which resulted in a decrease of the cumulative cell number by more than 50% 48 hours after the induction of differentiation (Figure 3B). Furthermore, hemoglobinization was reduced by 40% (Figure 3C). Benzidine stainings of these erythroblasts confirmed the differences regarding cell size distribution and hemoglobin accumulation (Figure 3D).

Taken together, these data show that 3% oxygen supplied by the used incubators interfere with normal terminal erythroid differentiation in our *in vitro* culture system.

p53 -/- erythroblast are more sensitive to low oxygen concentrations during terminal erythroid differentiation than wild type erythroblasts

Immortalized normal erythroblast clones isolated from fetal livers of p53-/- mice are characterized by reduced spontaneous differentiation when kept at non-differentiating conditions but behave otherwise like wt fetal liver erythroblasts[25]. We used such a clone (I/11) to further analyze the effect of oxygen on the proliferation and the differentiation of erythroblasts, since these cells do not show spontaneous differentiation and the switch between immature self renewal and terminal differentiation is strictly dependent on the factors added. Reduced oxygen levels had similar effects on the differentiation of I/11 erythroblasts compared to wild type fetal liver erythroblasts. The cell size decrease was normal at 20% oxygen: 72h after induction of differentiation a major part of the population reduced its diameter to 5 µm (Figure 4A, left panel). As expected, 3% oxygen interfered with cell size decrease but interestingly, the effect was even stronger compared to wild type erythroblasts (Figure 4A, right panel). The whole population of I/11 cells reduced the cell size only slightly each day. After 72 hours the mean diameter was still above 7 µm and cells with a diameter of 5 µm were virtually absent. Cell proliferation was also affected (Figure 4B). While I/11 erythroblasts increased the cell count more than 6 fold within a time span of 72 hours when cultivated at 20% oxygen, 3% oxygen reduced the increase to 2.5 fold. Furthermore, proliferation stopped after 48 hours indicating that differentiation was not only delayed but completely blocked. In addition, the mean heme amount/cell volume was reduced to 30% when the oxygen levels were reduced to 3% oxygen during differentiation (Figure 4C). Taken together, I11 cells are a

perfect tool to further evaluate the effect of oxygen concentration and of parameters linked to oxygen supply on erythroid differentiation.

Cell density and diffusion distance modulate the oxygen microenvironment of differentiating erythroblasts

The oxygen concentration in the direct environment of *in vitro* cultured cells is dependent on the oxygen concentration in the incubator. Nevertheless, it is often neglected that the cell density, the height of medium and the metabolic rate of the used cell type play an important role regarding the oxygen supply of the cell[15]. In order to investigate if the erythroblasts cultivated in our in vitro setting sense an oxygen environment that corresponds to the oxygen concentration of the incubator we challenged the system by manipulating factors that are known to influence oxygen availability.

First, we modulated the oxygen consumption rate per culture plate by reducing the number of erythroblasts seeded in the same volume of medium on an unchanged area. Proliferation rates of cells that were cultivated under self-renewing conditions were markedly affected by cell density (Figure 5A). When I/11 erythroblasts were readjusted daily to a concentration of 1x10E6 cells/ml, the cells grown at 3% oxygen showed a 50% decreased proliferation rate compared to cells that were grown at 20% oxygen. When the cell concentration was reduced by a factor of 4, the difference of the oxygen concentration did not influence the proliferation rates any longer. Interestingly the proliferation rate of cells cultivated at 3% oxygen at low density was comparable to the proliferation rate of cells seeded at high density in a 20% oxygen incubator. This demonstrates that the effective oxygen concentration in the environment of the proliferating erythroblasts in our *in vitro* system can be reduced by a high cell concentration.

When we looked at differentiating erythroblasts we could observe that the cell size decrease was perturbed by low oxygen levels (Figure 4A). This effect was still present when we reduced the cell concentration from 2x10E6 to 5x10E5 cells/ml (Figure 5B, left panel). Surprisingly, further reduction to 1.25x10E5 cells/ml erased any difference between the cultivation at 3 or 20% oxygen and the cell size distributions of erythroblasts 72 hours after the start of differentiation were practically equal (Figure 5B, right panel). The efficiency of heme accumulation was also dependent on oxygen concentration when differentiation was initiated at 2x10E6 cells/ml but this dependency diminished when the concentration was reduced, reaching comparable levels at 5x10E5

cells/ml and below (Figure 5C). Interestingly, the heme synthesis rate generally increased when the cell concentration became lower, indicating that heme synthesis was limited at higher cell concentrations. Comparable results were obtained when we looked at the proliferation rate during differentiation (Figure 5D). The observed reduction in proliferation caused by lower oxygen supply of the incubator at higher cell densities diminished with lower cell densities and was absent at a concentration of 1.25x10E5 cells/ml. Taken together, our results clearly show that the cell density modulates oxygen availability during the culture of erythroid cells leading to lower effective oxygen supply than the actual external oxygen concentration provided by the incubator.

Another oxygen-supply relevant parameter that we changed was the diffusion distance of oxygen through the medium that covers the cells. It is possible to reduce the distance to virtually zero if gas permeable tissue culture dishes are used[15]. Furthermore, other possible limiting factors that could be dependent on cell density can be neglected since these cultivation factors remain constant. When we cultivated I/11 erythroblasts in gas permeable dishes, we observed an effect that was comparable to a reduction of cell density. The proliferation rate of self renewing (Figure 6A) and terminal differentiating cells (Figure 6D) was similar in normal tissue culture dishes at 20% oxygen and in gas permeable dishes at 3% oxygen in contrast to the reduced proliferation rate in conventional tissue culture dishes at 3% oxygen. Interestingly, the proliferation rate of immature erythroblasts in gas permeable dishes at 20% oxygen was higher than in conventional dishes at the same oxygen concentration (Figure 6A). The heme synthesis rate was also positively affected by the usage of gas permeable dishes at 3% oxygen (Figure 6C). The cell size decrease could be improved but was still not as fast as with 20% oxygen (Figure 6B). However, erythroblasts that were cultivated in gas permeable dishes at 20% oxygen showed a comparable retardation in cell size decrease (data not shown). This indicates that this is an inherent effect of the gas permeable dishes and not due to a limited supply of oxygen.

2-oxoglutarate–dependent dioxygenase inhibitor DMOG prevents immature erythroblast from spontaneous differentiation *in vitro*

Since the cultivation of erythroblasts at low oxygen levels resulted in a reduction of spontaneous differentiation we wanted to further investigate the mechanisms behind this observation. HIFs are the regulators of the transcriptional response to hypoxia in metazoan organisms. In mammalians their activity is dependent on the hydroxylation of

three residues of their α-subunits (either HIF1α or HIF2α), which leads to the subunit's deactivation and degradation[26-28]. As this process is oxygen dependent, HIFs are active in a hypoxic environment. A simple possibility to activate HIFs despite high oxygen concentrations is by adding the 2-oxyglutarate-dependent dioxygenase inhibitor DMOG to cultured cells[27]. DMOG replaces 2-oxoglutarate, which is an essential co-substrate for HIF hydroxylation and therefore prevents HIF inactivation, since DMOG cannot be used by the relevant hydroxylation enzymes (PHDs, FIH). We used DMOG in order to investigate the role of HIFs in the regulation of erythroid differentiation.

The effects of DMOG on the outgrowth of fetal liver erythroblasts were not only comparable to the incubation at low oxygen levels but resulted in an even more pronounced effect. While the population of untreated control cells proliferated and differentiated simultaneously as shown above, DMOG treated cells proliferated but did not show any tendency to differentiate as demonstrated by cell size distribution (Figure 7A). This period of exclusive proliferation could be extended to at least 2 weeks, when DMOG was added regularly to the medium.

Using flow cytometry we further looked at the expression levels of the cell surface markers CD117/c-Kit and CD71/TfR1 that change during the process of erythropoiesis (Figure 7B). CD117, the receptor of the stem cell factor, is generally expressed on immature hematopoietic cells[29]. At day 7 of cultivation we could observe that the majority of erythroblasts that were grown in medium containing DMOG expressed CD117. In contrast to this, the greater part of cells without DMOG grown at 20% oxygen showed no CD117 expression, demonstrating that these cells are more mature than DMOG treated cells. CD71 is expressed on virtually every growing cell as iron import is essential for cell growth and proliferation. Regarding erythroblasts, its expression is strongly increased during terminal differentiation due to the high heme synthesis rate which is linked to high iron demands. As expected, erythroblasts cultivated with DMOG showed a lower expression of CD71 when compared to cells cultivated without DMOG, supporting the assumption that DMOG treated cells are more immature. Although CD71 expression is generally accepted as erythroid differentiation marker, its expression is also regulated by iron metabolism and consequently also linked to heme synthesis per se. Benzidine staining of day 7 erythroblasts showed that heme synthesis was strongly reduced by DMOG (Figure 7C, upper panels). Furthermore, the untreated population consisted of erythroblasts at different stages of maturation ranging from immature cells with large nuclei to small, mature, heme containing and even enucleated cells. In contrast to this, the DMOG treated population consisted mainly of a homogenous population of non-

differentiated cells. Taken all these data together, i.e. cell size distribution, the expression rates of erythroid markers, cell morphology and heme synthesis, this demonstrates that the addition of DMOG to the cultivated erythroblasts kept these cells in a more immature state than control cells.

In order to find out how an immature continuously DMOG treated population reacts to DMOG withdrawal we divided an 8 days old homogenous population of fetal liver derived erythroblasts cultivated with DMOG into a cell population with prolonged DMOG treatment and a population cultivated without DMOG. Cells that were supplemented with DMOG continued to proliferate without signs of spontaneous differentiation for at least 10 days (Figure 7D, middle panel). Interestingly, erythroblasts without DMOG showed an increase of the mean sell size of about 1 µm during the first 24 hours of DMOG withdrawal (Figure 7D, left panel). Thereafter the cells continuously reduced their mean cell size to 5 µm within 10 days. The cell size decrease was accompanied by an increase in the total proliferation rate until day 7 after DMOG withdrawal (Figure 7E). When we discriminated between the accumulating cell numbers of larger, immature cells (>7.5 µm diameter) and smaller, mature cells (4-7.5 µm diameter), we could observe that the higher proliferation rate of erythroblasts without DMOG was mainly caused by an increase in the number of small mature cells. In contrast, the relative amount of larger self renewing cells decreased, which consequently led to an increased “aging” of the population and finally to a stop of proliferation due to progressive depletion of immature erythroblasts. The decrease of cell size was accompanied by an increase of heme levels confirming terminal erythroid differentiation (Figure 7F; left panel). Both observations were confirmed by cytospin stainings. A major part of erythroblasts cultivated without DMOG was stained by benzidine indicating accumulation of heme and these cells also showed a cell size decrease which was accompanied by condensation of the nucleus or even enucleation (Figure 7C; lower panels). Erythroblasts that were grown in medium containing DMOG did not show this shift to more mature cells and continued to proliferate.

After about three weeks in culture DMOG treated cells showed a less defined cell size distribution with somewhat increased proportions of smaller cells. Interestingly, at the same time proliferation rates began to decrease. Furthermore, the heme levels remained low, indicating that the cells did not differentiate. In order to test if these erythroblasts are actually still able to undergo terminal differentiation, we again divided the DMOG treated population into two cultures with and without prolonged DMOG treatment at day 19. The effects of DMOG withdrawal were comparable to the effects at day 8, including cell size decrease (Figure 7D, right panel), increased proliferation and

heme synthesis rates (Figure 7F, right panel). However, the kinetic of spontaneous differentiation was accelerated and proliferation stopped within 5 days (Figure 7E). This indicates that although terminal differentiation was blocked by DMOG, the erythroid population became more inclined to differentiate if this block was removed.

DMOG interferes with induced terminal erythroid differentiation

The addition of DMOG to erythroblasts kept under non-differentiating conditions resulted in the extension of their proliferation potential linked to an abrogation of spontaneous differentiation when compared to cells without DMOG. Thus, we wanted to know whether there is an effect of DMOG on erythroblast induced for terminal differentiation.

For this purpose we cultivated fetal liver cells under self renewal conditions in the presence of DMOG until we established a homogenous population of immature erythroblasts. Subsequently we induced terminal erythroid differentiation by changing the supplemented factors and cultivated them further with or without the addition of DMOG.

When we measured the cell size distribution it was obvious that differentiating cells that were cultivated in the presence of DMOG reduced their cell size to a lesser extent than control cells without DMOG (Figure 8A). This observation was supported by cytospins of erythroblasts 48 and 72 hours after induction of differentiation (Figure 8B). Untreated cells showed a normal reduction in cell size including condensation of the nucleus and enucleation. In contrast to this, DMOG treated cells showed only a minor reduction of cell size and enucleation could not be observed. Furthermore, even 72h after induction of terminal erythroid differentiation heme synthesis was not detectable by benzidine staining in contrast to control cells without DMOG. Photometric determination of heme levels indeed showed that heme synthesis of DMOG treated cells was only slightly above or even beyond detection limits throughout differentiation, whereas control cells showed a normal increase of heme levels over time (Figure 8D). Interestingly, DMOG also negatively affected proliferation rates, resulting in a reduction of cell number by 50% 72 hours after induction of differentiation (Figure 8C). Finally, we also analyzed the expression of CD71 and Ter119, two known markers of terminal erythroid differentiation, by flow cytometry. The expression of both was increased on differentiated control cells as well as on DMOG treated cells compared to immature erythroblasts (Figure 8E). However, the increase was weak in DMOG treated cells compared to control, supporting an inhibitory effect of DMOG on terminal erythroid differentiation.

5.3.5. Discussion

The activation of epo transcription via stabilization of HIF-2α in the kidney links hypoxia/oxygen-supply to erythropoiesis. The direct contribution of the oxygen concentration to the development of red cells in the erythropoietic tissues itself is less well understood. Furthermore, the regulation of the cellular iron metabolism, which is vital during erythropoiesis in order to supply the heme synthesis machinery with sufficient amounts of iron, is also affected by oxygen. The aim of this work was to analyze the effects of cultivation-dependent parameters on oxygen supply and furthermore, the direct effect of the oxygen concentration on erythropoiesis, including the regulation of the cellular iron metabolism by the IRE/IRP system.

We could show that a reduction of the external oxygen supply had no influence on the binding activities of IRPs as long as erythroblasts were kept in an immature state. Nevertheless, when we induced terminal differentiation, which is accompanied by a drastic increase in heme biosynthesis and a considerable reduction in cell size, the IRP/IRE system responded to low oxygen concentrations by reducing IRP binding activity. This would be in conflict with an increased iron demand during this period of erythropoiesis, since reduced IRP binding activity leads to reduced iron import via TfR1 and increased sequestration of iron by ferritin[30]. Indeed, the incubation of erythroblasts at low oxygen concentrations resulted in reduced heme levels. Surprisingly, we also observed disturbed cell proliferation and cell size decrease during induced differentiation at 3% oxygen compared to atmospheric oxygen levels. Apparently, reduced oxygen levels interfered with the whole process of terminal differentiation of the cultivated erythroblasts suggesting that the reduction of IRP binding activity was a secondary effect, caused by the inhibition of heme synthesis and the resulting iron overload[31]. This is supported by the fact that we could not observe a change in IRP binding activity in more immature erythroblasts, i.e. before the induction of heme synthesis.

The occurrence of an inhibitory effect of low oxygen levels on erythroid differentiation was very surprising, since physiological oxygen concentrations in mammalian tissues are normally in the range of 3 to 5% or even lower, depending on oxygen supply and metabolic activity[11]. It is generally assumed that *in vivo*-like tissue culture experiments should be performed at oxygen concentrations corresponding to the physiological levels, especially if oxygen dependent processes are analyzed. Nevertheless, several groups have shown that the effective oxygen concentration that is

perceived by *in vitro* cultured cells is not equivalent to the oxygen concentration of the incubator but is also dependent on other factors, like cell density, metabolic rate or diffusion distance[15]. We could show that the cell density modulates the oxygen availability in our *in vitro* system and our results suggest that the effective oxygen concentration perceived by erythroblasts cultivated at atmospheric oxygen levels is in fact below 20%. Furthermore, when we used gas permeable dishes that eliminate the limiting effects of oxygen diffusion through the medium, we detected no difference between erythroblasts cultured at 3% oxygen and conventional cultivation at 20% oxygen, indicating that the oxygen supply of the cells is comparable. This implies that under conventional culture conditions combined with normal atmospheric oxygen supply the effective oxygen concentration is similar to physiological conditions during terminal erythroid differentiation. Consequently, when the oxygen concentration is decreased to 3% the effective oxygen supply is further decreased and reaches hypoxic levels that interfere with the late stages of differentiation. This was still the case when the oxygen concentration was adjusted to less "hypoxic" levels of 6% oxygen (data not shown).

Since HIFs are the transcription factors that regulate the cellular response to reduced oxygen supply, we wanted to analyze if they are also responsible for the observed effects on erythropoiesis. To test the role of HIFs, we added the HIF stabilizer DMOG to cultured erythroblasts. Surprisingly, the outcome was even more pronounced than with cells cultured under low oxygen levels. This observation can be explained by our method of cultivation, which includes the daily addition of fresh medium containing the respective factors. Hence, it was necessary to interrupt the cultivation in the low oxygen incubator and to expose the erythroblasts to atmospheric oxygen levels every 24 hours. Therefore, the activity and stabilization of HIF-α is interrupted as well and has to be reestablished after the medium change leading to a gap in the activation of its targets. In contrast to this, DMOG is present continuously and activates HIFs without interruption, independent of exposure to oxygen and medium changes.

As already described, the used erythroid *in vitro* model includes two phases of cultivation[17]. In the first phase, immature erythroblasts are cultivated in the presence of factors promoting self renewing with only a minor tendency for spontaneous differentiation. In the second phase, they are replaced by factors inducing synchronous terminal differentiation, which finally leads to a population of small, enucleated and highly hemoglobinized cells. In both phases, reduced oxygen supply as well as DMOG treatment inhibited cell size decrease, enucleation and heme synthesis, which resulted in a homogenous immature population in the first phase and perturbed terminal differentiation in the second phase of the cultivation. Obviously, low oxygen supply and

stabilization of HIF-α support self renewal of immature erythroblasts and interfere with terminal erythroid differentiation.

Furthermore, the addition of DMOG to fetal liver cells not only prevented the terminal differentiation of erythroblasts, which resulted in a homogenous population of immature cells during the first phase of cultivation, but also prolonged their limited capacity to proliferate. The cell size distribution of the cultured cells already indicates that cells without DMOG become more mature within several days and the proportion of immature cells with still higher proliferation capacity decreased. Recently it has been shown that the expansion rate of purified BFU-Es but not of more mature CFU-Es can be increased by the addition of DMOG and it was demonstrated that HIFs and glucocorticoids, which are essential for stress erythropoiesis, share transcription targets[16]. Addition of DMOG to fetal liver cells would therefore result in an accumulation of BFU-Es in our culture system. This is in line with the observation that 24 hours after DMOG withdrawal erythroblasts increase their mean cell volume, which would confer with the transition of smaller BFU-Es to larger CFU-Es[32]. The BFU-Es isolated from fetal livers normally are able to proliferate for 10 days, which corresponds to the proliferative capacity of this cell type[33,34]. Nevertheless, our total fetal liver culture could be kept proliferating for at least 28 days, indicating that beside BFU-Es more immature cells with still higher proliferation capacity can be found in the cultivated population. These early erythroid progenitors would therefore be responsive to hypoxia and HIFs as well. Another explanation would be that the self-renewing capacity of BFU-Es themselves is prolonged under the influence of hypoxia.

Although terminal erythroid differentiation was virtually absent throughout the incubation with DMOG, SCF and dex, the erythroblasts obviously became more disposed to differentiation during cultivation. While the population of fetal liver cells depleted of DMOG at day 8 of cultivation, needed about 10 days until all cells were fully differentiated, erythroblasts that were already three weeks in culture fully differentiated within 5 days as soon as DMOG was withdrawn. Additional characterization of cultivated erythroblasts treated with DMOG will help to clarify in detail how oxygen and HIF influence the different stages of erythropoiesis.

Based on these data we can conclude that the oxygen concentration in combination with growth factors effectively and directly regulates the progression of erythroid differentiation and that the activity of HIFs is responsible for this oxygen dependent regulation. This is in perfect agreement with the *in vivo* situation, since the oxygen concentration in hematopoietic tissues increases in line with increasing maturity

of the developing erythroblasts[12]. Therefore, cultivation of maturing erythroblasts requires adjustment of the oxygen concentration during the process of terminal differentiation in order to assure *in vivo* like conditions.

Since low oxygen concentrations inhibited the last phase of erythroid differentiation, we are confronted with a seemingly paradox situation. Hypoxia generally leads to an increased formation of fully developed erythrocytes mainly induced by an increase of epo synthesis in the kidney[9,10,35]. A direct inhibitory effect of hypoxia on erythroid differentiation would therefore contradict the observed amplification of erythrocytes. Based on our data, we want to propose a model that clarifies this paradox and furthermore demonstrates how hypoxia contributes to the increased generation of erythrocytes directly in the hematopoietic tissue. We show that there is an oxygen threshold that is essential for the decision between self renewal and differentiation. This threshold divides the hematopoietic tissue into a hypoxic area, which promotes self renewal and contains immature cells, and a normoxic area that allows terminal erythroid differentiation. If an organism is confronted with reduced oxygen supply, this will lead to a shift of the threshold leading to an enlargement of the hypoxic area. Hence, developing erythroblasts reside in the hypoxic area for a longer time period and therefore are able to stay in a self renewal state and divide more often before the onset of terminal erythroid differentiation compared to an organism that is sufficiently supplied with oxygen. This leads to an increase in the proliferation rate and consequently to an increased number of mature erythrocytes, since terminal differentiation can still take place in the more oxygenated regions. Thus, in our model hypoxia induces erythropoiesis indirectly via the induction of epo and directly via the oxygen distribution at the site of erythropoiesis in order to guarantee increased generation of erythrocytes.

The promoting activity of dioxygenase inhibitors like DMOG on the early stages of erythropoiesis shows promising potential for a therapy addressing certain non epo responsive types of anemia[16]. Nevertheless, a possible therapy by the administration of these inhibitors must take into account that the site of early erythropoiesis is already hypoxic[12]. An additional activation of HIFs would indeed increase the proliferation rate of BFU-Es and more immature erythroid progenitors, but at the same time terminal erythroid differentiation would be perturbed. *In vivo* experiments including determination of oxygen concentration and expression of HIFs would therefore help to evaluate a possible therapy.

Taken together, we could show that cultivation parameters like cell density and diffusion distance have to be considered, when oxygen is an important factor of *in vitro*

experiments. Furthermore, the oxygen concentration can be used as a regulator of erythroid development *in vitro* and is an important factor in the cultivation of erythroblasts. Finally we present a model, describing how hypoxia can contribute *in vivo* to induce erythropoiesis directly, additional to the activation of epo expression in the kidney.

5.3.6. Figures

Figure 1

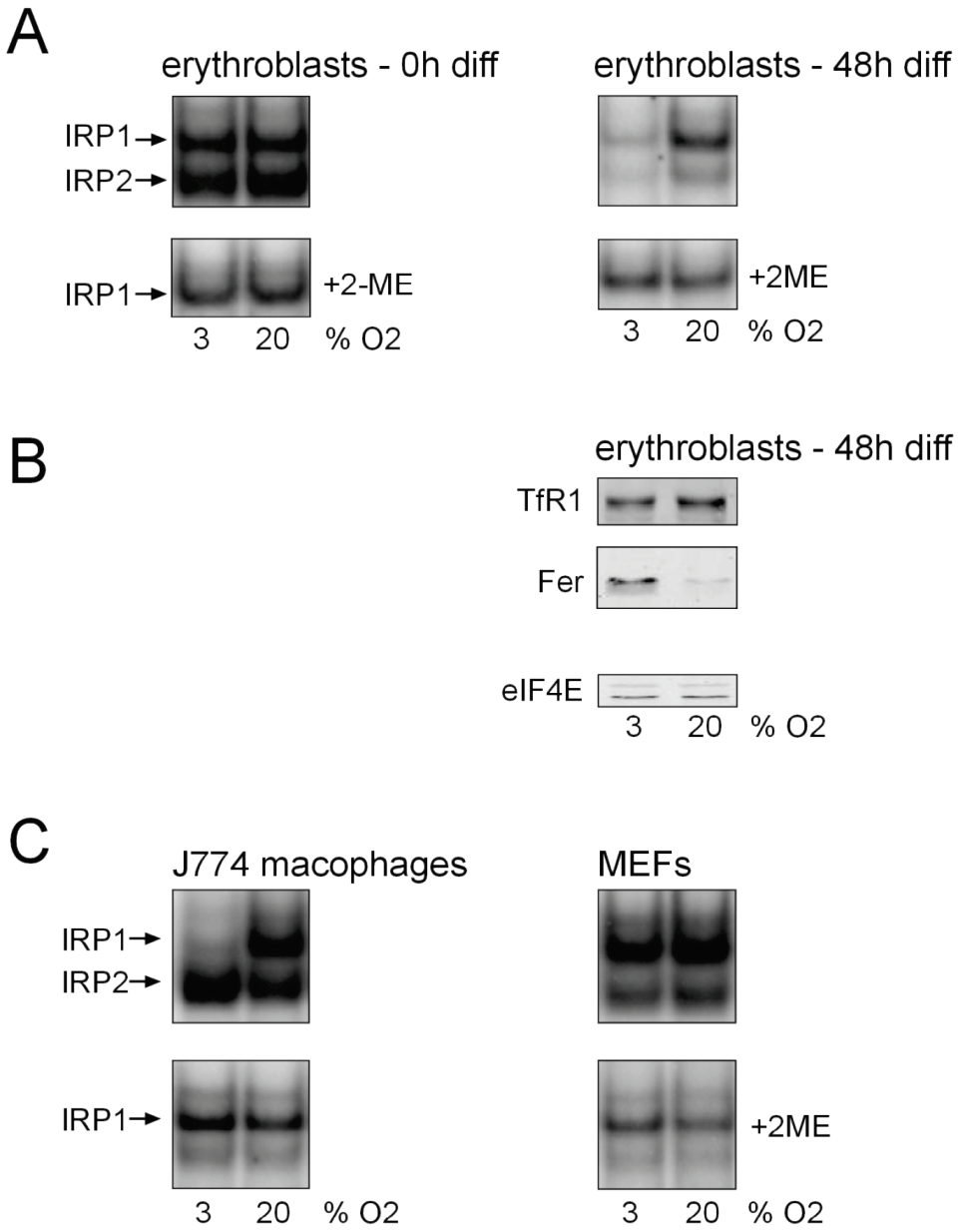


Figure 1. The response of the iron regulatory system to changed oxygen levels is cell type specific. (A) Cytosolic extracts of *in vitro* cultured immature and differentiating erythroblasts (48 hours after induction of differentiation) were used for EMSA to analyze the binding activity of IRP1 and IRP2 at different oxygen conditions (3% and 20%). 2-mercaptoethanol treated lysates were used as loading control. (B) The protein levels of TfR1 and Fer of the cell lysates described above were determined by western blot analysis. eIF4E was used as loading control. (C) Cytosolic extracts of J774 macrophages and MEFs cultivated at hypoxic and normoxic conditions were analyzed for IRP binding activity.

Figure 2

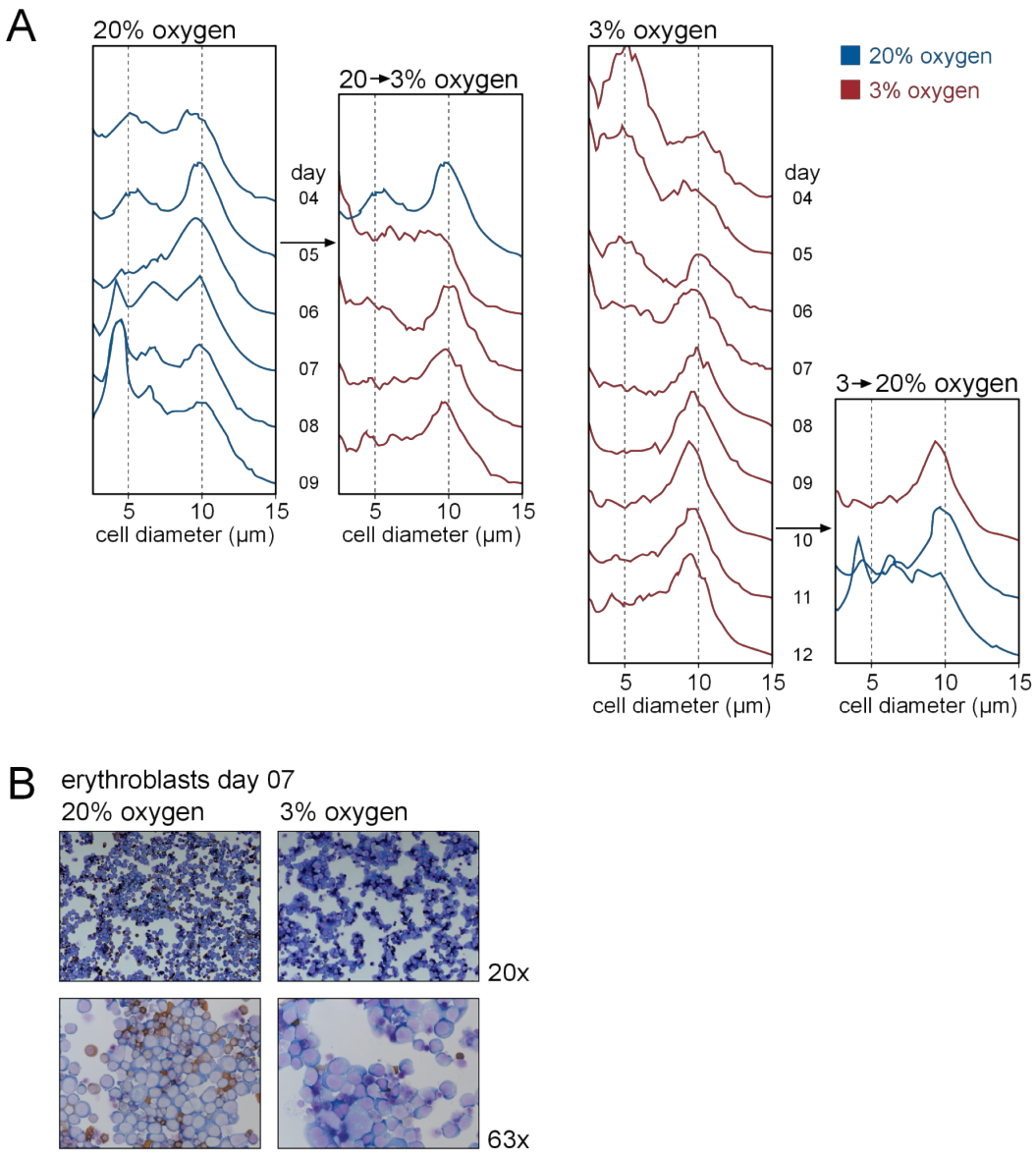


Figure 2. Low oxygen prevents spontaneous terminal differentiation of immature erythroblasts. Murine fetal liver cells were cultivated in medium containing SCF, dex and epo at 3% and 20% oxygen. (A) Cell size distribution was monitored daily with an electronic cell analyzer starting 4 days after the onset of cultivation. At day 5, a fraction of the erythroblasts cultivated at 20% oxygen was transferred to 3% oxygen and at day 10 erythroblasts cultivated at 3% oxygen were transferred to 20% oxygen. Immature erythroblasts have a mean diameter of 9 to 11µm whereas mature differentiated erythroblasts show a diameter of 5µm. (B) Seven days after starting the cultivation of fetal liver cells, cultured erythroblasts were cytocentrifuged onto glass slides and stained with benzidine and histological dyes. Decrease of cell size is accompanied by condensation of the nucleus and enucleation. Benzidine stain of hemoglobin containing cells results in a brown colored cytosol.

Figure 3

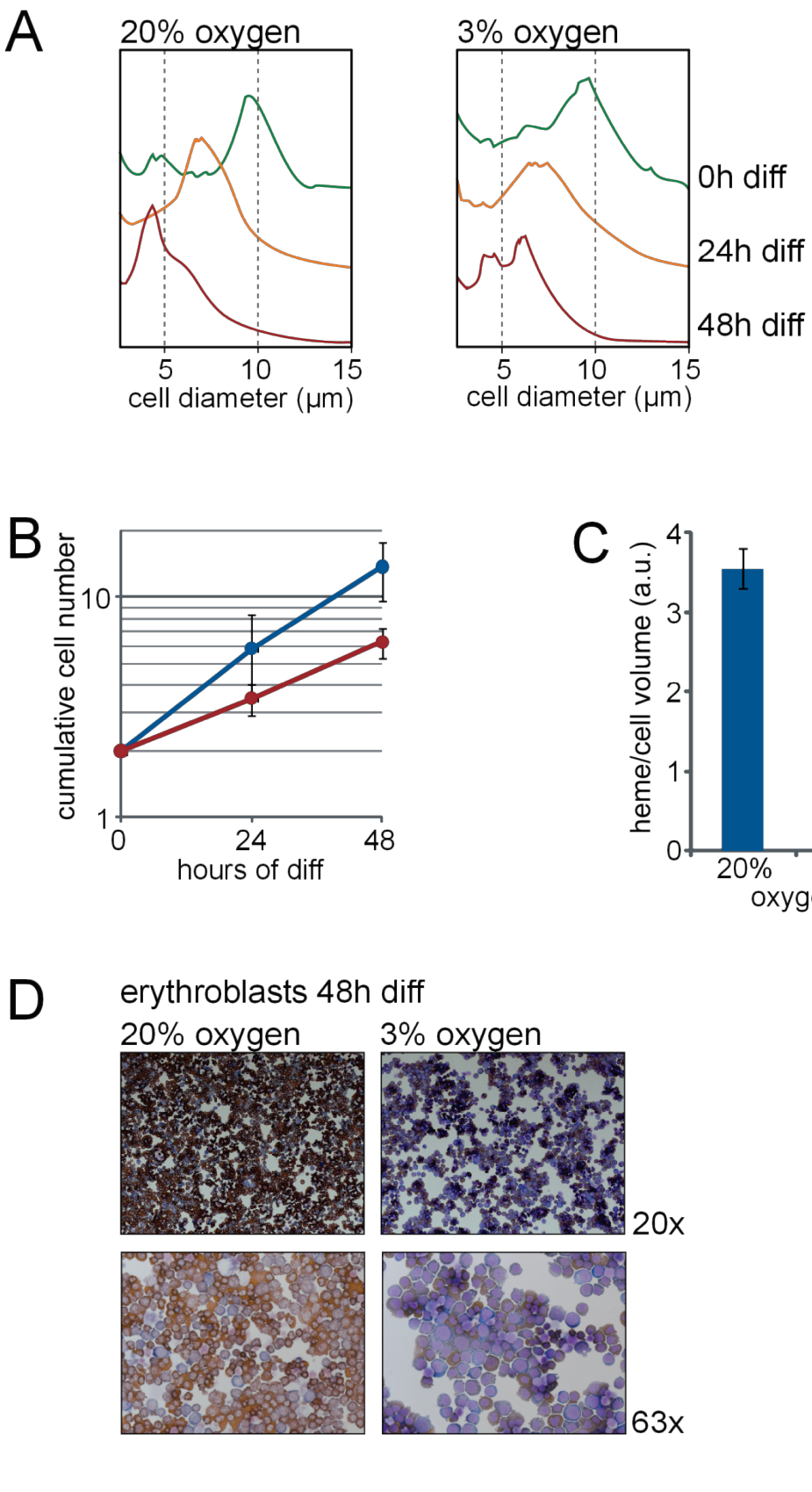

A
20% oxygen
3% oxygen
0h diff
24h diff
48h diff
5
10
15
cell diameter (µm)
B
cumulative cell number
10
1
0
24
48
hours of diff
C
heme/cell volume (a.u.)
4
3
2
1
0
20%
3%
oxygen
D
erythroblasts 48h diff
20% oxygen
3% oxygen
20x
63x

Figure 3. Hypoxia perturbs normal induced terminal differentiation of erythroblasts. Immature erythroblasts were cultivated under self renewal conditions for seven days at 20% and 3% oxygen and consequently switched to medium containing differentiation factors. (A) Cell size distribution was determined starting with the onset of differentiation. (B) Cumulative cell numbers of differentiating erythroblasts were calculated as described in material and methods; error bars indicate SD of mean, n=4. (C) Heme/cell volume of erythroblasts 48 hours after induction of differentiation was determined as described in material and methods; error bars indicate SD of mean, n=3. (D) Benzidine staining of terminally differentiating erythroblasts 48 hours after induction.

Figure 4

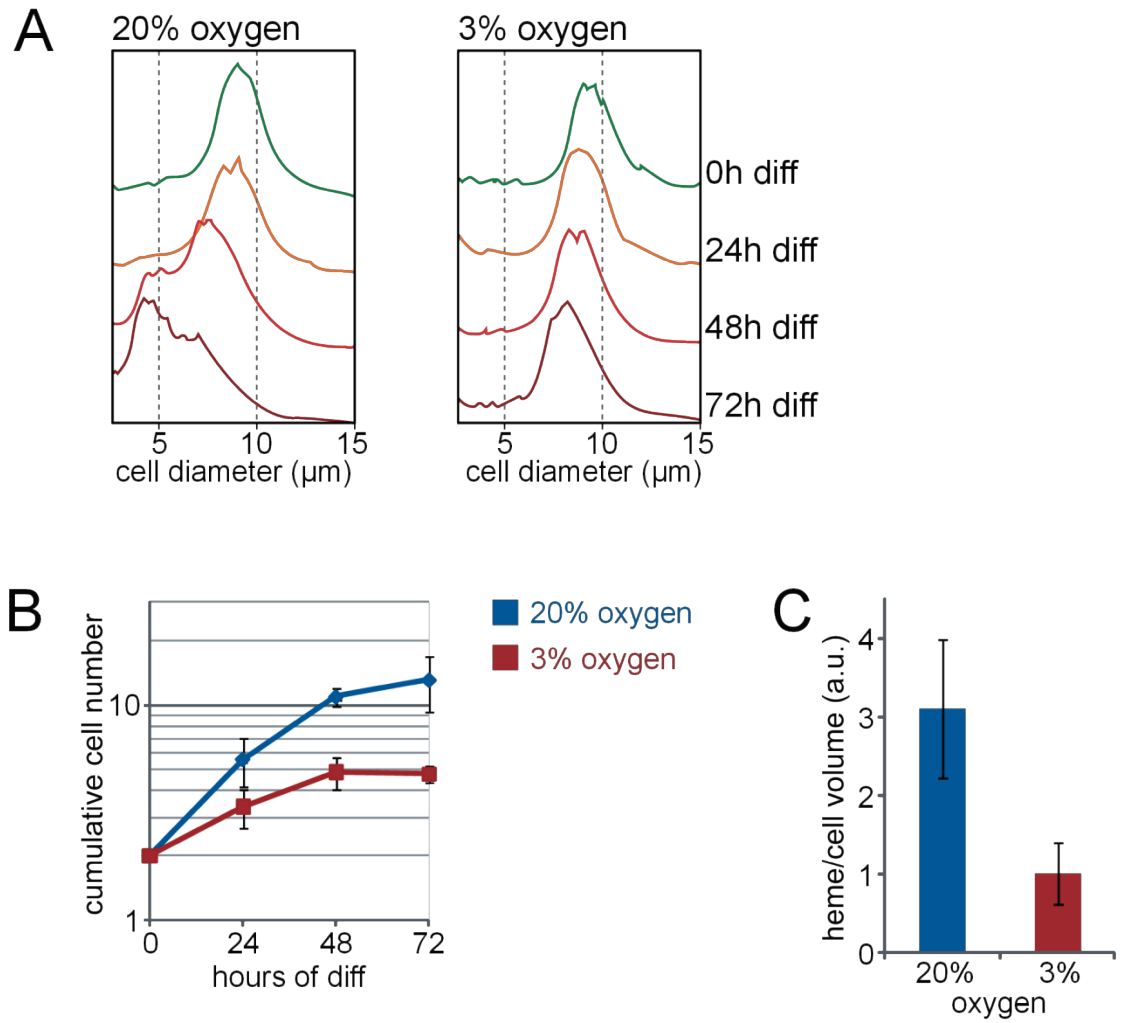


Figure 4. p53 -/- erythroblast are more sensitive to low oxygen concentrations during terminal erythroid differentiation than wild type erythroblasts. p53 -/- fetal liver derived erythroblasts were induced to terminal differentiation. (A)Cell size distribution of these cells was measured every 24 hours starting with induction of terminal differentiation. (B) Cumulative cell numbers and (C) heme levels of differentiating p53 -/- erythroblasts; error bars indicate SD of mean, n=3.

Figure 5

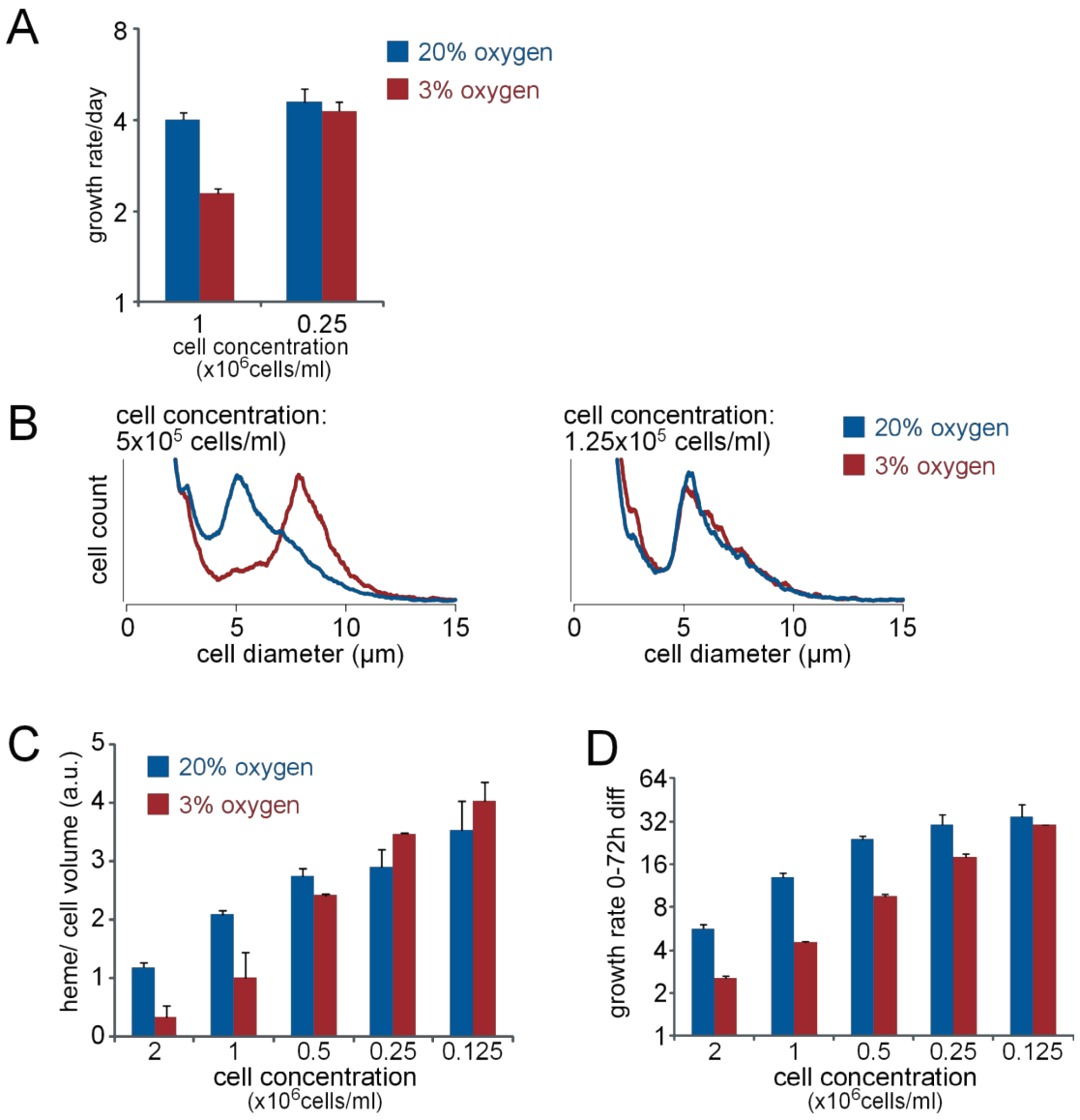


Figure 5. Cell density modulates the oxygen microenvironment of cultivated p53-/- erythroblasts. We cultivated p53 -/- fetal liver derived erythroblasts at different cell and oxygen concentrations and monitored the outcome on proliferation and differentiation. (A) Growth rate/day of self renewing erythroblasts cultivated at 3% and 20% oxygen at the indicated cell density; error bars indicate SD of mean, n = 3 (B) Differentiating erythroblasts were cultivated at the indicated cell densities and cell size distribution, (C) heme levels and (D) growth rates were determined 72 hours after induction of differentiation; error bars indicate SD of mean, n=3.

Figure 6

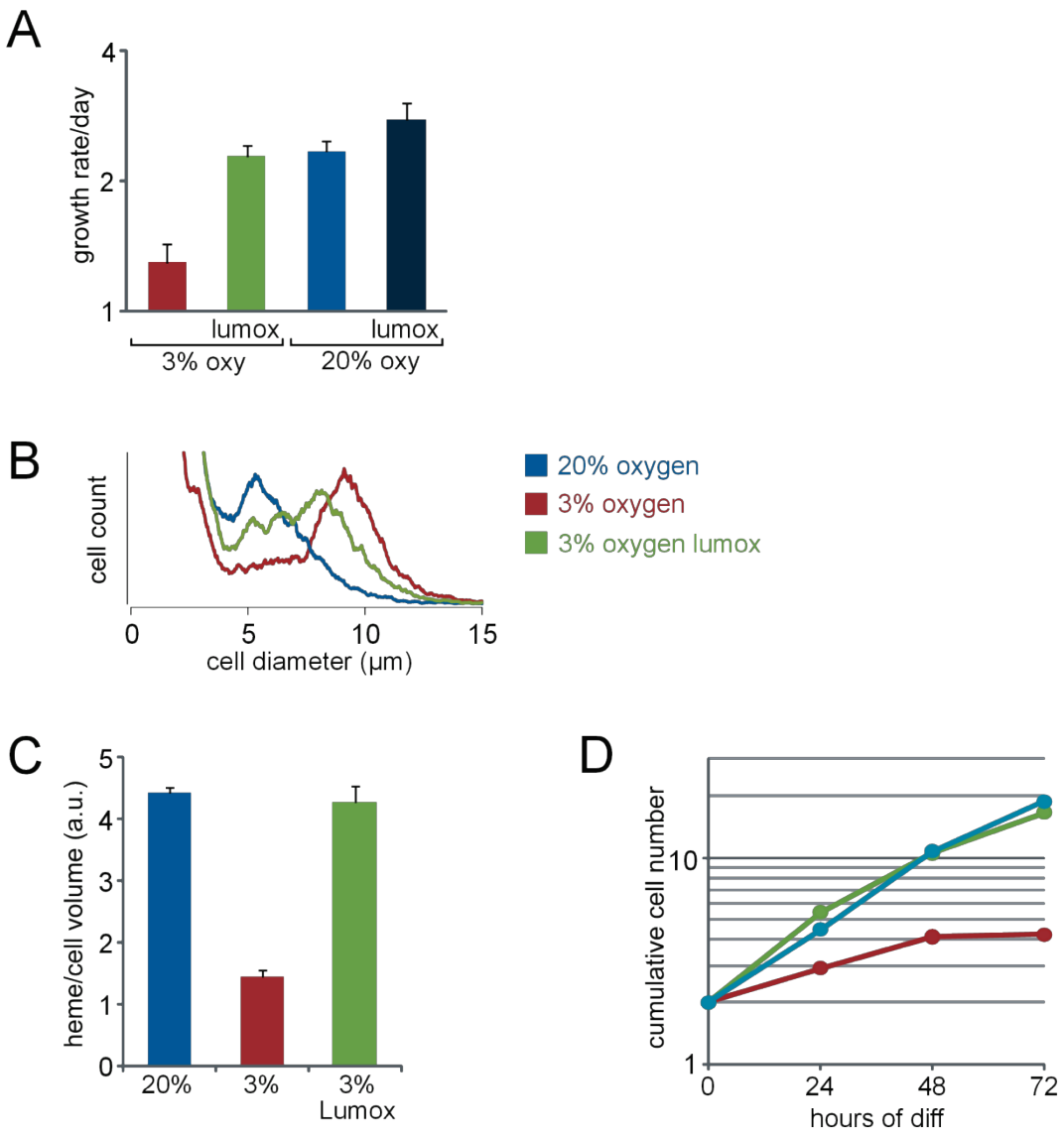
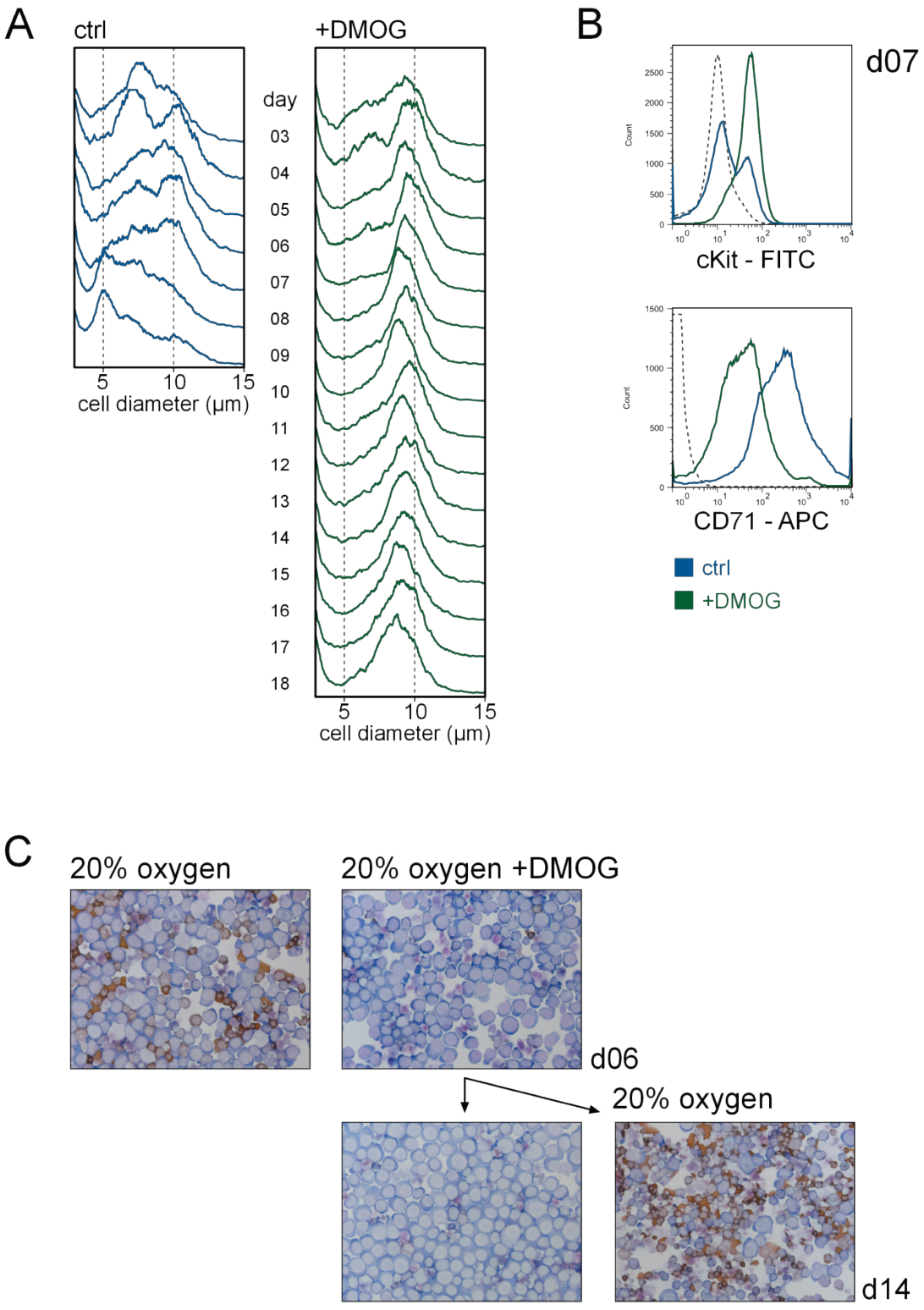
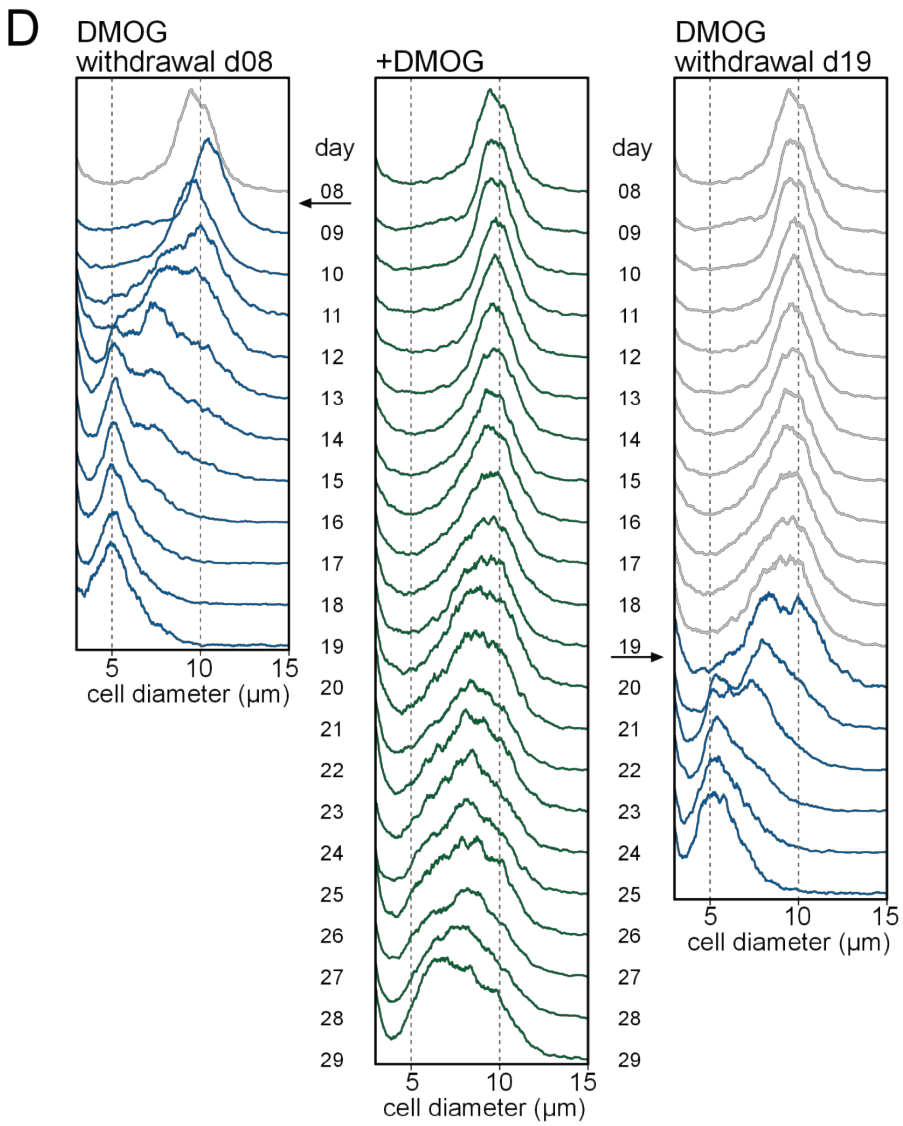


Figure 6. The diffusion distance limits the oxygen availability for cultured erythroblasts. p53 -/- fetal liver derived erythroblasts were cultivated in conventional and gas permeable tissue culture plates (Lumox). (A) Self renewing erythroblasts were cultivated at 3% and 20% oxygen and the daily proliferation rate was determined; error bars indicate SD of mean, n = 5. (B) Differentiating erythroblasts were cultivated as indicated and cell size distribution and (C) heme levels were determined 96 hours after induction of differentiation. (D) Cell numbers were determined every 24 hours; error bars indicate SD of mean, n=3.

Figure 7


A
ctrl
+DMOG
day
03
04
05
06
07
08
09
10
11
12
13
14
15
16
17
18
5
10
15
cell diameter (µm)
B
d07
cKit - FITC
CD71 - APC
ctrl
+DMOG
C
20% oxygen
20% oxygen +DMOG
d06
20% oxygen
d14


D
DMOG
withdrawal d08
+DMOG
DMOG
withdrawal d19
day
08
09
10
11
12
13
14
15
16
17
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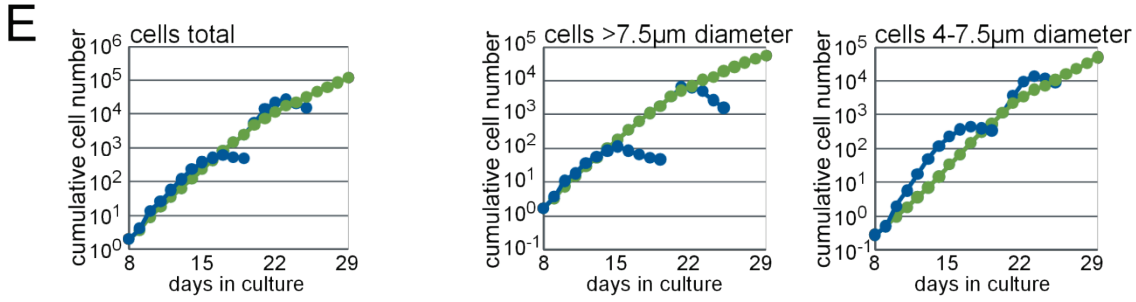
22
23
24
25
26
27
28
29
5
10
15
cell diameter (µm)


E
cells total
cells >7.5µm diameter
cells 4-7.5µm diameter
cumulative cell number
days in culture

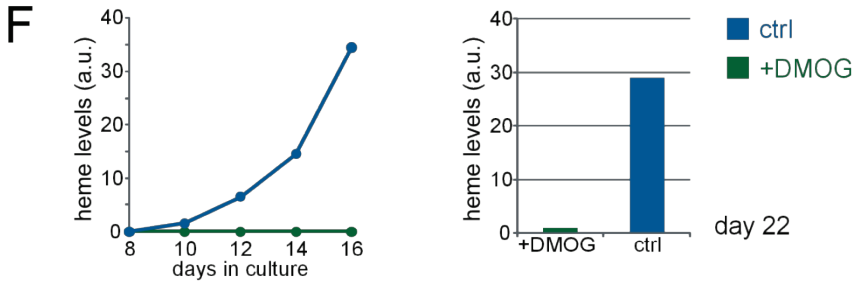

F
heme levels (a.u.)
days in culture
ctrl
+DMOG
day 22

Figure 7. 2-oxoglutarate–dependent dioxygenase inhibitor DMOG reduces spontaneous differentiation and extends the proliferation capacity of immature erythroblasts. Fetal liver cells were cultivated at 20% oxygen in the presence or absence of DMOG. (A) Cell size distribution was monitored daily with an electronic cell analyzer, starting 3 days after the onset of cultivation. (B) Flow cytometry histograms of fetal liver cells cultivated for 7 days, stained for CD117/c-kit and CD71/TfR1. (C) Benzidine staining of erythroblasts cultivated for 6 days (upper panel) with or without DMOG. The erythroblasts initially cultivated with DMOG were further cultured in the presence or absence of DMOG until day 14 (lower panel).

(D) A homologous, DMOG treated population of fetal liver cells was divided into two separate populations at day 8 of cultivation. One population was further supplemented with DMOG while the other population was cultivated without DMOG. This procedure was repeated at day 19. We determined cell size distribution, (E) cumulative cell numbers and (F) heme levels of these cell populations.

Figure 8

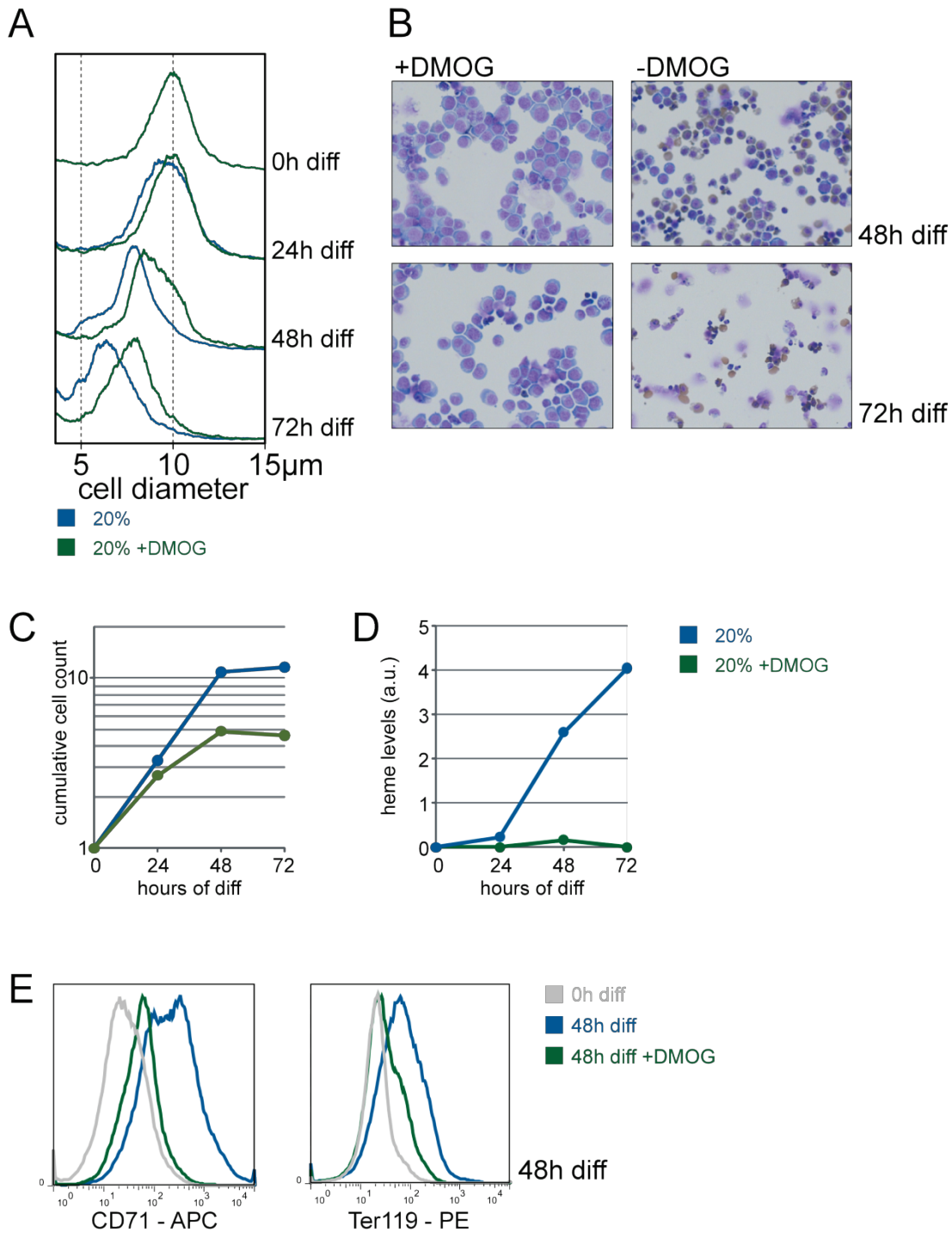


Figure 8. DMOG interferes with induced terminal erythroid differentiation. Immature erythroblasts were cultivated under self renewal conditions and consequently switched to medium containing differentiation factors including or excluding DMOG. (A) Cell size distribution was determined daily, starting with the onset of differentiation. (B) Benzidine staining of terminally differentiating erythroblasts 48 and 72 hours after induction of

differentiation. (C) Cumulative cell numbers and (D) heme levels of differentiating erythroblasts. (E) Flow cytometry histograms of erythroblasts differentiating for 48 hours, stained for CD71/TfR1 and Ter119. Immature erythroblasts were used as control.

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6. Discussion

6.1. The regulation of iron metabolism in differentiating erythroblasts

The proper acquisition of iron for heme synthesis is essential for the successful differentiation of erythroblasts, since reduced iron supply results in the development of anemia. Nevertheless, it is not sufficient that an adequate amount of iron-bound transferrin provided by the systemic iron household is available for heme synthesis. In fact, the differentiating erythroblasts must be able to handle the large amounts of iron that need to be imported, transferred to the mitochondria and finally incorporated into protoporphyrin IX in order to produce heme for the formation of hemoglobin. We are interested in how the cellular IRE/IRP system regulates the expression of ferritin, ALAS2 and TfR1, three main components of the erythroid iron metabolism, in order to ensure an unobstructed heme synthesis. Theoretically, induced heme synthesis requires increased iron import via TfR1 and minimal iron sequestration by ferritin. Furthermore, ALAS2 expression must be elevated in order to allow the synthesis of sufficient amounts of protoporphyrin IX. This is in perfect agreement with our observations when we examined the expression rates of these proteins in differentiating erythroblasts. Nevertheless, this contradicts the expected regulation pattern of the IRE/IRP system: Increased iron accumulation within a cell normally decreases the iron binding activity of IRPs, which consequently leads to the downregulation of TfR1 and the upregulation of ferritin[47,59]. Surprisingly, this expression pattern was not only turned upside down after the induction of terminal differentiation but also did not respond to the modulation of the iron household by the addition of the iron chelator desferrioxamine. In contrast to the observed discrepancies between expected regulation by the IRE/IRP system and observed expression of TfR1 and ferritin, ALAS2 translation was not only induced during differentiation but also still responded to the addition of desferrioxamine. Nevertheless, this comprises another inconsistency since due to the IREs in the 5' UTRs of both, ALAS2 and ferritin, their expression is expected to be regulated synchronously.

Thus, what are the mechanisms that explain the discrepancy in the expression between erythroblasts before and after the onset of differentiation? In fact, the increased import of iron is combined with increased iron processing during the production of heme. Although the exact path of iron from the endosomes to the mitochondria is still not known, it is obvious that iron is shuttled very fast to the site of heme synthesis.

Consequently, we hypothesize that the residence time of iron within the cytosol is reduced to a minimum leading to a virtually iron depleted environment. This results in an increase of IRP binding activity and explains the observed increase of TfR1 expression as well as the decreased translation of ferritin mRNA. Furthermore, the insensitivity to desferrioxamine treatment is explained by the fact that the cytosol is already iron depleted and hence the addition of the chelator is without effect. We challenged this hypothesis by artificially increasing the cytosolic iron concentration of cultured erythroblasts using FAC as additional iron source. Indeed, ferritin expression correlated with the used iron concentration indicating the full functionality of the IRE/IRP system. Moreover, inhibition of heme synthesis by the addition of INH or SA activated ferritin expression as well. This demonstrates that under normal conditions mitochondria use iron instantaneously for heme synthesis and as a result they deprive the cytosol of iron. Nevertheless, this still does not explain the differences between Fer and ALAS2 expression. We hypothesize that two reasons are responsible for this discrepancy. First, although all IREs share common properties, it has been shown that due to differences in structure and sequence their binding affinity for IRPs varies[147,148]. Second, the presence of two IRPs that bind to specific IREs with different affinities allows a certain degree of fine-tuning of the regulatory system. Furthermore, the IRP1 binding activity is dependent on the iron sulfur cluster synthesis and becomes activated if the mitochondrial iron supply is reduced. In contrast to this, IRP2 stability is dependent on the presence of cytosolic iron. Therefore, IRP1 activity is linked to mitochondrial iron supply while IRP2 mediates adaption to cytosolic iron availability respectively iron excess. In combination, these features allow a more specific regulation of IRE containing mRNAs and might explain the observed differences between ferritin and ALAS2 expression.

The generation of IRP1 and IRP2 deficient mice demonstrated that despite a basic redundancy of IRP activity, allowing survival without either one of the two IRP homologues, there are obvious differences in their phenotypes[166]. In general, IRP1 deficiency is linked to only minor changes of iron distribution, whereas IRP2 knock out mice show several pathological effects including an erythroid phenotype. The observed microcytic anemia of IRP2 deficient mice correlates with reduced TfR1 expression in bone marrow cells and therefore it was proposed to be caused by reduced iron import of differentiating erythroblasts[167,168]. We further addressed this question by using IRP1-/- and IRP2-/- fetal liver cells. In line with the anemic phenotype of IRP2 deficient mice, differentiated IRP2-/- erythroblasts showed reduced heme levels compared to control cells demonstrating that anemia is indeed an intrinsic erythroid effect. Interestingly, TfR1 expression of IRP2 deficient erythroblasts was reduced before the onset of heme

synthesis compared to control cells but the expression returned to normal levels during terminal differentiation, indicating that IRP independent mechanisms may contribute to TfR1 upregulation. In contrast, ferritin expression was elevated in IRP2-/- erythroblasts throughout the cultivation before and after the onset of heme synthesis. Based on these data we conclude that the upregulation of ferritin leads to the sequestration of iron in IRP2-/- erythroblasts and therefore is the main cause for the observed microcytic anemia in IRP2 deficient mice. Since TfR1 is expressed at high levels during the period of heme synthesis its contribution to the phenotype is of minor importance.

Taken together, cultivated primary erythroblasts derived from IRP deficient mice are a perfect tool to investigate special aspects of iron metabolism during erythroid differentiation. For instance, further analysis will reveal the contribution of IRP1 and IRP2 to the regulation of ALAS2 activity and thereby will clarify the discrepancy between ferritin and ALAS2 regulation mentioned above.

6.2. The impact of oxygen on the regulation of erythropoiesis

Oxygen has been shown to influence the binding activity of IRPs[155,272,273]. Furthermore, the oxygen concentration has an impact on differentiation in general and on erythropoiesis in particular, although the underlying mechanisms are not well understood[261,266]. Since we use a cell culture system in order to analyze the iron metabolism during erythropoiesis these oxygen dependencies are of special importance for our work. As a consequence, the application of conventional incubators supplied with atmospheric oxygen levels imply that the oxygen concentration is unphysiological compared to the in vivo situation. The reduction of the oxygen concentration to “physiological levels” (3-5% oxygen) indeed affected the IRP binding activity in terminally differentiating erythroblasts but surprisingly did not alter the binding activity in immature erythroblasts before the onset of heme synthesis. Furthermore, cultured fibroblasts showed no response to changes in oxygen supply, while macrophages responded, although the changes were distinct from those observed with differentiating erythroblasts. Consequently, we conclude that the effect of the oxygen concentration on the IRP binding activity is dependent on additional indirect factors. In the case of differentiating erythroblasts we could show that reduced oxygen in general had an inhibiting effect on terminal differentiation. In combination with SCF and glucocorticoids this resulted in the establishment of a homogenous population of immature erythroblasts with a minor tendency to spontaneously differentiate than cells cultivated at atmospheric

oxygen levels. On the other hand, the induction of terminal differentiation by the withdrawal of SCF and dex and the increase of epo concentration was inhibited by reduced oxygen levels which resulted in reduced proliferation, perturbed cell size decrease and reduced heme synthesis. As mentioned above, inhibition of heme synthesis during terminal erythroid differentiation increases cytosolic iron levels and leads to the downregulation of IRP binding activity. Taken together, this explains why the limited oxygen supply decreases the binding activity of both IRPs and why the IRPs of immature cells are not responsive to reduced oxygen since the differentiation-linked heme synthesis is not yet induced in these cells.

The average oxygen concentration in tissues is normally in the range from 3% to 5%, but locally it is even lower[183]. Compared to these values the atmospheric oxygen concentration of 20-21% seems far too high. As a consequence some cell culture experiments are performed at oxygen concentrations that correspond to the *in vivo* levels in order to allow physiological conditions, especially if oxygen dependent processes are analyzed. Nevertheless several papers that addressed the question of oxygen supply in conventional tissue culture could show that the effective oxygen concentration is not equivalent to the ambient oxygen concentration[267,269,271]. Moreover, the limitations of oxygen diffusion in combination with high oxygen consumption rates can lead to a virtually anoxic environment for certain cell types[268,271]. We modulated the cell density and reduced the diffusion distance in our cell culture system in order to find out whether the cultivated erythroblasts face oxygen concentrations that are below the levels provided by the used incubators. As mentioned above, the reduction of the ambient oxygen concentration to 3% resulted in the inhibition of erythroid differentiation under conventional culture conditions. The reduction of the cell density that at the same time reduces the overall oxygen consumption led to a complete elimination of this effect. Similar results were achieved by reducing the diffusion distance using gas permeable dishes. Hence, our data clearly show that the conventional culture conditions result in a decrease of the effective oxygen concentration in the direct environment of the erythroblasts compared to the ambient levels. Furthermore, we can assume that under modulated culture conditions the diffusion to consumption ratio increases and consequently the effective oxygen concentration approximates the levels supplied by the incubator. This illustrates that an effective oxygen concentration of 3% still allows normal erythroid differentiation and only lower levels interfere with the maturation of these cells.

The link between erythropoiesis and oxygen levels corresponds to the *in vivo* situation in hematopoietic tissues, where the increase of maturity correlates with an increase of oxygen concentration[266,274]. Our data show that oxygen is able to actively

regulate the ongoing of erythropoiesis, which allows its fine tuning during a hypoxic situation. A systemic oxygen undersupply not only induces epo production in the kidney, but also increases the low-oxygen area in the hematopoietic tissues. Consequently, this leads to a prolonged proliferation period of erythroblasts before the onset of terminal differentiation and, in combination with increased epo levels, eventually results in an increased number of mature erythrocytes.

Since our data show that the oxygen concentration directly regulates the development of erythroblasts we were interested in the mechanisms that are responsible for this effect. Promising candidates that might be involved in the regulation of erythropoiesis are the HIF proteins, which are responsible for the main part of the transcriptional response to reduced oxygen supply. 2-oxoglutarate dependent oxygenases induce the inactivation and degradation of the HIF-α subunits if oxygen is present, but these enzymes can be inhibited by the addition of 2-oxoglutarate analogs like DMOG[207]. The addition of DMOG to cultured erythroblasts indeed resulted in the same effects that we observed at reduced oxygen concentrations, including reduced heme synthesis, cell size decrease and proliferation. Moreover, the proliferation capacity of immature erythroblasts could be extended even longer than with low oxygen supply. Therefore, our data demonstrate that the inhibition of 2-oxoglutarate dependent oxygenases is sufficient to reproduce hypoxic conditions. Recently, Flygare and coworkers presented comparable results that show a positive effect of DMOG on the proliferation of cultivated BFU-Es, although they report increased proliferation in contrast to prolonged proliferation as presented in our work and they do not describe an inhibiting effect of DMOG on terminal erythroid differentiation[275]. They could show that glucocorticoids and HIF share common target genes and therefore propose that this redundancy is responsible for the effect on erythroid proliferation. Nevertheless, it cannot be excluded at the moment that oxygenases not involved in the regulation of HIF-α may contribute as well. Currently we are generating erythroid cells that express a stable and active version of HIF-1α in order to clarify these uncertainties.

Taken together we could show that the oxygen concentration directly affects erythropoiesis. Furthermore we present a model that incorporates these observations in the systemic response to hypoxia. Finally, we demonstrated that the underlying mechanism is dependent on the activity of 2-oxoglurarate dependent oxygenases. Further analysis of the involved HIF-regulated genes will contribute to a better understanding of the direct contribution of oxygen concentration to the regulation of erythropoiesis.

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9. Curriculum vitae

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Career related activities

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Publications

Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1,

Kerenyi MA, Grebien F, Gehart H, **Schifrer M**, Artaker M, Kovacic B, Beug H, Morrigl R, Müllner EW;

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Schranzhofer M, **Schifrer M**, Cabrera JA, Kopp S, Chiba P, Beug H, Müllner EW;

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Poster Presentations (selection)

Hypoxia and its role in the maturation of red blood cells

Schifrer M, Schranzhofer M, Beug H, Müllner EW

Molecular and Applied Biosciences Austria, 2010 September 27-29, Vienna, Austria

Studying iron metabolism in erythroid cells under different oxygen conditions

Schifrer M, Schranzhofer M, Müllner EW

BioIron, 2009 June 7-11, International BioIron Society meeting, Porto, Portugal

Distinct functions of iron regulatory proteins during terminal differentiation of primary erythroid cells

Schifrer M, Schranzhofer M, Müllner EW

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Modulation of IRP binding activity by oxygen in primary erythroid cells

Schifrer M, Schranzhofer M, Müllner EW

BioIron, 2007 April 1-6, International BioIron Society meeting, Kyoto, Japan

European Iron Club meeting, 2007 September 13-15, London, UK

Delta aminolevulinic acid synthase and the regulation of iron homeostasis in normal erythroblasts

Schifrer M, Schranzhofer M, Cabrera JA, Müllner EW

BioIron, 2005 May 22-25, International BioIron Society meeting, Prague, Czech Republic