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The Role of Iron in Benign and Malignant Hematopoiesis

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Abstract

Significance: Iron is an essential element required for sustaining a normal healthy life. However, an excess amount of iron in the bloodstream and tissue generates toxic hydroxyl radicals through Fenton reactions. Henceforth, a balance in iron concentration is extremely important to maintain cellular homeostasis in both normal hematopoiesis and erythropoiesis. Iron deficiency or iron overload can impact hematopoiesis and is associated with many hematological diseases.

Recent Advances: The mechanisms of action of key iron regulators such as erythroferrone and the discovery of new drugs, such as ACE-536/luspatercept, are of potential interest to treat hematological disorders, such as β-thalassemia. New therapies targeting inflammation-induced ineffective erythropoiesis are also in progress. Furthermore, emerging evidences support differential interactions between iron and its cellular antioxidant responses of hematopoietic and neighboring stromal cells. Both iron and its systemic regulator, such as hepcidin, play a significant role in regulating erythropoiesis.

Critical Issues: Significant pre-clinical studies are on the way and new drugs targeting iron metabolism have been recently approved or are undergoing clinical trials to treat pathological conditions with impaired erythropoiesis such as myelodysplastic syndromes or β-thalassemia.

Future Directions: Future studies should explore how iron regulates hematopoiesis in both benign and malignant conditions. *Antioxid. Redox Signal.* 35, 415–432.

Keywords: iron, hematopoiesis, erythropoiesis, oxidative stress

Introduction

The unique properties of iron are essential to many physiological phenomena responsible for sustaining life. Iron can readily accept or donate an electron to participate in oxidation–reduction reactions, such as those that occur in cellular respiration, nucleic acid synthesis, metabolic reactions, and oxygen transport. Iron is also crucial for mitochondrial biogenesis, heme synthesis, and the formation of iron–sulfur clusters, which are essential electron-transfer proteins (98, 119). However, the same properties that give iron its versatility in orchestrating a wide array of diverse physiological processes present a danger. In the human body, iron uptake and its concentration are stringently regulated as there are no mechanisms for excreting iron from the body once absorbed. Therefore, the balance between iron uptake, transport, utilization, and storage must be regulated in a very precise and orderly manner.

Excess iron is toxic and can form free radicals leading to oxidative stress, DNA, and tissue damage. Failure to properly regulate systemic iron levels can lead to impairment of many biological processes, giving rise to a wide range of pathological conditions, including anemia and iron overload-related disorders. Iron accumulation has been shown in genetic disorders

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and hematological and neurodegenerative diseases (61). In this review, we discuss the different regulatory pathways involved in iron metabolism as it relates to hematopoiesis. We have also emphasized the perturbations of iron homeostasis in hematological disorders such as hemochromatosis, β-thalassemia, and anemia of inflammation (AI). Finally, we summarize novel therapeutic interventions that can restore iron homeostasis in patients suffering from iron deficiency- or iron overload-related hematological disorders.

Systemic Iron Homeostasis

Uptake, export, and recycling of iron

Iron enters the body from the diet. Approximately 2 mg of iron are absorbed by enterocytes every day after being reduced from ferric (Fe^{3+}) to ferrous state (Fe^{2+}) by duodenal cytochrome B-reductase also known as duodenal cytochrome *b* (113). Divalent metal transporter 1, an iron importer with 12 transmembrane domains localized at the apical membrane of enterocytes, transports Fe^{2+} into the intracellular space (66). The excess iron is stored in ferritin, the universal storehouse of iron. Ferritin is a highly conserved globular protein consisting of 24 subunits and forms a central cavity where iron is stored (20). During iron deficiency, iron is released from ferritin. A selective nuclear receptor coactivator 4 (NCOA4) mediating the release of iron was recently identified (15). The process by which NCOA4 releases iron from ferritin is known as ferritinophagy. Ncoa4 knockout (KO) mice fed an iron-rich diet died prematurely due to iron overload and exhibited signs of severe liver damage. In contrast, Ncoa4-KO mice receiving a low-iron diet developed microcytic hypochromic anemia due to inefficient mobilizing of iron from ferritin stores (15).

Ferrous iron (Fe^{2+}) is highly reactive and toxic and is therefore oxidized back to the ferric state (Fe^{3+}) by the ferroxidase hephaestin (169). It enters circulation through ferroportin (FPN), the only known exporter of elemental iron in the cell (43, 124, 128). Fe^{3+} iron in the bloodstream is then transported by the carrier protein, transferrin (Tf). The majority of this iron is used in the synthesis of heme and iron–sulfur clusters in the mitochondria. Tf binds to its receptors, transferrin receptor 1 (TFR1) and receptor 2 (TFR2). $Tfr1^{-/-}$ mice are embryonically lethal at E12.5 due to severe anemia (100). However, mice lacking TFR1 only in hepatocytes are viable although showing relatively high levels of hepcidin (when normalized to liver iron content), modest hypoferremia, and microcytosis. This indicates that TFR1 is redundant for basal hepatocellular iron supply but essential for fine-tuning hepcidin responses, most likely through its interaction with HFE (47, 149).

TFR1 is also known to be highly expressed in erythroid cells, brain, skeletal muscle cells, gut cells, and cardiomyocytes, whereas TFR2 is known to be only expressed in the brain, kidney, liver, colon, and testes (13, 36, 175). Recent studies have shown a novel iron-sensing function of TFR2 in erythropoiesis (123). TFR2 binds to and stabilizes the erythropoietin (EPO) receptor (EPOR) in erythroid precursors (50). Studies have also identified another molecular pathway involving the scaffold protein scribble that links the iron-sensing function of TFR2 to EPOR expression at the cell membrane of erythroid precursors (91). Under low iron conditions, unbound TFR2 traffics to lysosomes and TFR2-scribble complexes are catabolized. As a consequence, the levels of scribble decrease and EPOR is not efficiently stabilized for surface presentation. Mouse chimeras with TFR2-deficient hematopoietic cells have erythrocytosis, increased EPO sensitivity, and reduced apoptosis of late erythroblasts (123).

Tf is a bilobed glycoprotein that binds one iron ion on each lobe. Tf and iron can associate in four distinct ways: (i) no iron bound (apo-Tf), (ii) iron bound on the lobe closest to the amino-terminal (Tf-N), (iii) iron bound on the lobe closest to the carboxyl-terminal (Tf-C) (both are mono-Tf), or (iv) iron bound to both lobes (holo-Tf). The two lobes have distinct affinities for iron (1, 71). Tf can be taken up by the cell *via* clathrin-mediated endocytosis after binding to TFR1 or TFR2. However, holo-Tf is thought to be the primary supplier of iron to differentiating erythroid cells. It has been also indicated that holo-Tf stabilizes TFR2 (86, 170). A new study shows that the different lobes of Tf mediate distinct signaling events within cells (129). In this study, two mouse models of mono-Tf were utilized. One model harbored a knockin mutation that inhibited iron binding on the N-terminal (Tf-N) lobe, while the other model had an analogous mutation, blocking iron binding to the C-terminal (Tf-C) lobe. It was observed that iron entering the cell *via* the Tf-N or Tf-C lobes affects EPO sensitivity distinctly.

In both models, animals were mildly anemic; however, the Tf-N mutants were unresponsive to exogenous EPO and had higher baseline levels of EPO in circulation. In contrast, Tf-C mutants had normal EPO levels and responded to exogenous EPO with greater sensitivity. Treatment of Tf-C mice with exogenous EPO produced more red blood cells (RBCs) compared with EPO-treated wild-type mice. To understand the molecular underpinnings of this finding, expression of protein kinase B/Akt was assessed, since this pathway is implicated in EPO responsiveness. Tf-N mutant mice had lower pAKT levels, suggesting that EPO responsiveness can be modulated by Tf forms. It is yet to be determined if these different sensitivities to EPO arise from Tf interactions with TFR1 or TFR2, as TFR2 is also found on early progenitors and has been implicated in EPOR stabilization at the membrane (50). Interestingly, both models exhibit a hemochromatosis phenotype despite having normal serum iron levels. The Tf-N mutant appeared mostly affected, showing higher liver iron loading and lower serum hepcidin compared with the C lobe mutant. This work highlights the importance of functionally distinct mono-Tf forms.

An illustration depicting iron intake, export, and recycling is shown in Figure 1.

In other models, under conditions of iron overload where the Tf saturation is high, excess free iron in the form of nontransferrin bound iron (NTBI) is present. NTBI is highly oxidative and leads to the formation of reactive hydroxyl radicals by Fenton reactions, resulting in oxidative stress (104). Cellular importers of NTBI during pathological conditions include zinc transporter proteins such as ZIP14 in hepatocytes and pancreatic acinar cells (78). This transporter together with ZIP8 has been identified as transmembrane proteins likely to regulate cellular uptake of divalent metal ions such as zinc, iron, manganese, and cadmium (79). Studies have shown that deficiency of Zip14 in mouse models can attenuate the conditions of iron overload in the liver and pancreas (78).

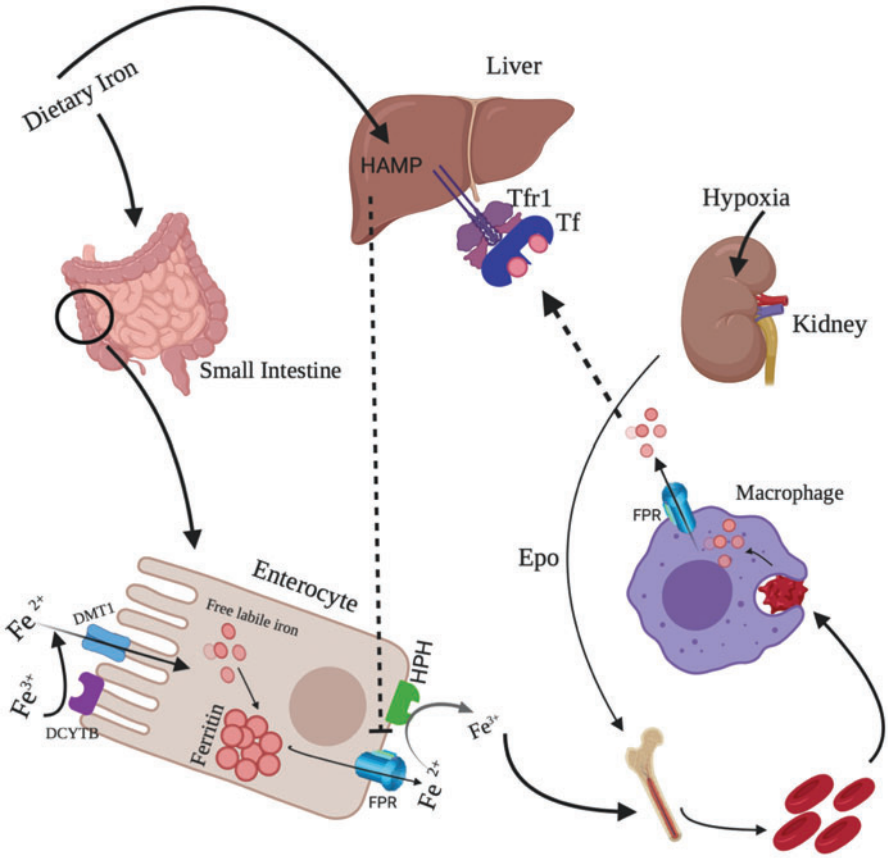

FIG. 1. Uptake, export, and recycling of iron. Dietary iron after being absorbed by intestinal cells is stored and then exported to the blood stream. Majority of the iron is utilized in the synthesis of RBC production under the stimulation of erythropoietin (a kidney hormone). Dead and senescent RBCs are phagocytosed by macrophages, resulting in the production of iron, which is again utilized for the heme production. RBC, red blood cell. Color images are available online.

Regulation of iron homeostasis

One of the most important regulators of iron homeostasis is hepcidin, a peptide synthesized by the liver that degrades FPN (43, 124, 128). The pathways that regulate the hepcidin gene (HAMP) are activated when iron rises to levels outside of homeostatic range, and are suppressed when iron concentrations fall (59, 61). Mutations in hepcidin and FPN are associated with hereditary hemochromatosis type 2b and type 4, respectively (125, 133, 145) (discussed in the section ''Steady-State Erythropoiesis''). Higher levels of hepcidin are implicated in several conditions, including AI (127). Significant players regulating hepcidin include the hemochromatosis-associated protein (HFE), hemojuvelin (HJV), bone morphogenetic proteins (BMPs), matriptase-2 (TMPRSS6), erythroferrone (ERFE), and interleukin 6 (IL-6). HJV is a coreceptor of BMPs (9, 10, 173).

The protein expression of HJV is detected in many tissues such as the liver, brain, heart, and skeletal muscle (144). Mutations in HJV lead to a severe iron-overload condition known as hemochromatosis type 2a (93, 99). Under high iron conditions, BMP2 and BMP6 in the liver initiate heterodimerization between BMP type I (ALK2/ALK3) and type II receptors, BMPRII/Act RIIA. This interaction results in the activation of the SMAD1/5/8 complex and SMAD4, which increases hepcidin expression (9). Liver sinusoidal endothelial cells are the primary source of BMP6 (28). Lack of BMP6 in mouse models leads to iron overload (3, 116). Furthermore, interaction of HFE with TFR2 stimulates hepcidin production (54).

The other important negative modulator of hepcidin is matriptase-2, also known as transmembrane II protease serine 6 (TMPRSS6). TMPRSS6 cleaves HJV and decreases hepcidin expression (154). In contrast, TMPRSS6 deficiency leads to increased hepcidin expression and, as a consequence, to iron refractory iron-deficiency anemia (48). However, in mice affected by β-thalassemia, TMPRSS6 deficiency improves iron overload and ineffective erythropoiesis (157). Another significant regulator of hepcidin is ERFE. It is secreted by erythroid progenitor cells under EPO stimulation and downregulates hepcidin in the liver (85). ERFE is also highly expressed in β-thalassemic mice. Mechanistically, it was proposed that the N-terminus of ERFE acts as a ligand trap for BMP6 and presumably downregulates hepcidin by sequestering BMP6 (5). A more recent study has shown that ERFE lowers hepcidin by sequestering a BMP2 and BMP6 heterodimer and prevents their binding to BMP type 1 ALK3 receptor (171). A receptor that binds ERFE has not yet been found.

In addition to the modulators described, hepcidin is also regulated by proinflammatory cytokines, namely IL-6, during inflammatory conditions such as autoimmune disorders, chronic infections, and cancer, characterized by AI. IL-6 stimulates hepcidin *via* the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway and limits plasma iron availability thereby promoting hypoferremia (52, 53). Additional studies have shown a regulatory role of BMP signaling in mediating IL6-JAK2-STAT5-mediated hepcidin induction (29). Furthermore, studies from our

laboratory have shown a role of both IL6 and hepcidin in AI (57). We are currently investigating the plausible reasons for how $Il6^{-/-}$ mice exhibit a better recovery in erythropoietic profile in the bone marrow (BM) from stress erythropoiesis when infected by a pathogen known as *Brucella abortus* (a mouse model of AI) (article in preparation by Sinha *et al.*).

The different regulatory pathways mediating iron homeostasis are shown in Figure 2.

Iron in Hematopoiesis

Iron and hematopoietic stem cells

Under physiological conditions, mitochondrial respiration represents the main source of reactive oxygen species (ROS), along with the actions of different enzymes such as NADPH oxidases. In iron-overload conditions, an increase in labile cellular iron leads to the generation of free radicals, culminating in cell damage or death, with consequent tissue damage. This typically occurs when Tf saturation exceeds 60%–70%, leading to the presence of NTBI in serum. NTBI ultimately leads to oxidative stress.

Recent studies suggest that hematopoietic stem cells (HSCs) residing in the BM are susceptible to iron overload, due to the generation of ROS that have a harmful impact on HSCs and their niche (Fig. 3). It has been proposed that patients with iron overload and impaired hematopoietic function may benefit from iron chelation (106). Tanaka *et al.* reported that *in vitro* iron treatment leads to ROS generation and ROS-mediated injury of both HSCs and differentiated hematopoietic cells (162). Remarkably, HFE hemochromatosis patients do not display a significant impairment in hematopoiesis despite the continuous iron accumulation throughout their lifetime (136). This is probably due to HSC protection within the BM *in vivo*, which is not replicated in an artificial *in vitro* coculture system with stromal cell lines.

Nevertheless, several *in vivo* studies using different mouse models suggest that the regulation of cellular iron is important for hematopoiesis. Mice injected with iron dextran have increased production of cellular ROS and impaired hematopoiesis (34). The F-box and leucine-rich repeat protein 5 (FBXL5) regulates cellular iron levels through the ubiquitination of iron regulatory protein 2 (IRP2). It was shown that Fbxl5-deficient HSCs have cellular iron overload that results in reduced cell numbers, impaired self-renewal, and stem cell exhaustion. Interestingly, HSCs from myelodysplastic syndrome (MDS) patients have reduced FBXL5 expression (122). The feline leukemia virus, subgroup C, receptor 1 (FLVCR1) is the only known heme exporter. It was shown that hematopoietic Flvcr1-deficient mice have blocked erythroid maturation and anemia, suggesting that erythroid progenitors require heme iron export for survival and differentiation (81, 87). Interestingly, a study demonstrated

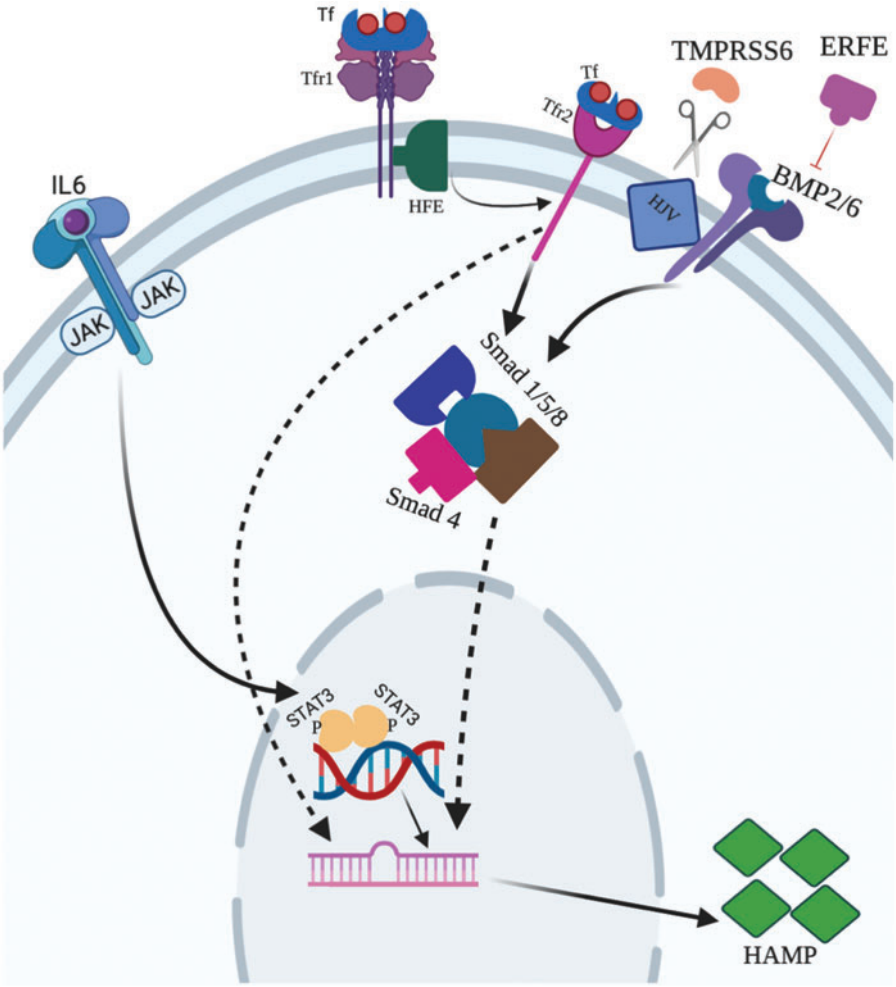

FIG. 2. Different regulatory pathways in the liver. Hepcidin, the master regulatory protein that regulates iron homeostasis, is modulated by different signals. Hepcidin is regulated by TFR1, TFR2, IL-6, and BMP signaling pathways. The negative regulators of this protein are TMPRSS6 and ERFE. TMPRSS6 is known to cleave HJV, whereas ERFE is known to act as a trap ligand of BMP. BMP, bone morphogenetic protein; ERFE, erythroferrone; HJV, hemojuvelin; IL-6, interleukin 6; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2; TMPRSS6, matriptase-2. Color images are available online.

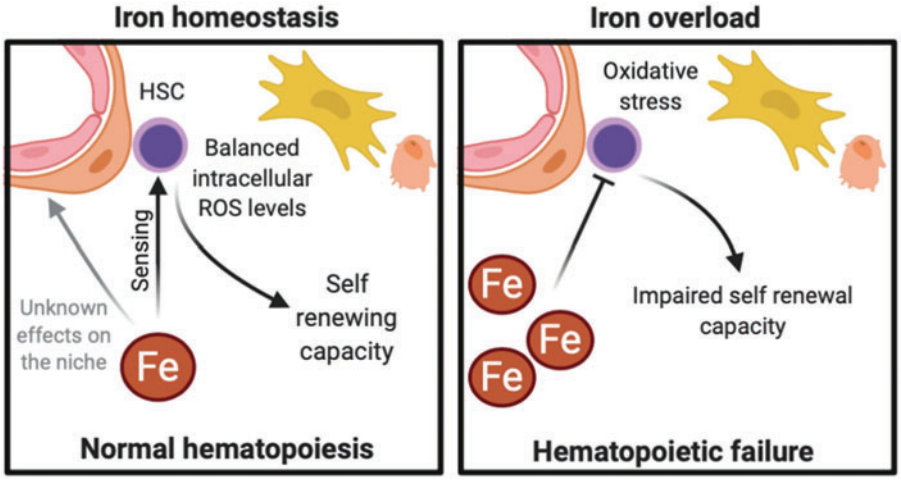

FIG. 3. The effect of iron on HSCs and their niche. While balanced intracellular oxidative stress levels are important in maintaining HSC function, iron overload increases ROS levels in HSCs and impairs their self-renewal. Iron is also thought to damage the HSC niche and impair HSC transplantation but their effect on the microenvironment is poorly studied. HSC, hematopoietic stem cell; ROS, reactive oxygen species. Color images are available online.

direct toxicity of iron and ROS on HSCs of mice with RUNX1-S291fs-induced MDS and iron overload (81). It should be noted, however, that extremely low levels of ROS can also hamper HSC function, causing defects in their differentiation and repopulation capacity (82). Altogether, it appears that finely tuned levels of iron are required to maintain HSC and hematopoietic homeostasis (180).

The impact of iron on hematopoiesis is also clinically demonstrated in patients undergoing hematopoietic stem cell transplantation (HSCT) who often develop iron overload as a consequence of frequent RBC transfusions. Postmortem studies of patients dying shortly after HSCT revealed levels of hepatic and BM iron comparable with the levels observed in patients with HFE hemochromatosis (158). Iron overload in HSCT may cause heart and pancreas damage and promote microbial infection (69, 95). It may also directly affect the BM niche and impair hematopoietic reconstitution, as suggested by the negative prognostic impact of pretransplantation elevated serum ferritin levels in HSCT outcomes (7).

Iron and the HSC niche

The BM microenvironment or niche comprises many different cell types and factors but are broadly characterized by perivascular and endosteal sites where self-renewing HSCs reside (132). Iron and ROS levels are important for both HSC and niche functions (107). Low ROS levels are key for maintenance of HSC quiescence, but increasing ROS are necessary for HSC differentiation (180). The interplay between ROS and the niche is well illustrated by the study of Itkin *et al.* (73). The authors demonstrated that HSCs localizing next to less permeable arterioles were immotile and in a quiescent state, while HSCs located close to more permeable sinusoids were migratory and differentiated (73). The importance of local iron differences within the BM and the role of iron exported by recycling macrophages in the regulation of ROS levels in HSCs remain to be explored. In the case of excessive oxidative stress, stem cell function is compromised as discussed and HSCs are biased toward a myeloid differentiation (75, 77). Ultimately, excessive ROS levels may lead to HSC exhaustion and apoptosis (74).

The cell-extrinsic role of iron overload and ROS in the BM niche is, however, poorly explored. Evidence from MDS studies suggests that iron overload may inhibit osteoblasts and increase osteoclasts' numbers and activity (24, 132). Using an acute myeloid leukemia (AML) mouse model, we have previously observed that the iron-chelator deferoxamine (DFO) protects the bone-lining endosteal vasculature and HSCs from leukemia-induced damage (44). This observation suggests that increased local iron may play a role in the remodeling of the niche in AML. Future studies should explore how iron affects HSC niche function.

NRF2 and hematopoiesis

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (NFE2l2 or NRF2) is the master regulator of the cellular antioxidant response (92, 109) and regulates the expression of detoxifying enzymes. NRF2 activity is controlled by the Kelch-like-ECH-associated protein 1 (KEAP1) that forms a complex with the Cullin-3-based E3 ubiquitin ligase and targets NRF2 for degradation through the ubiquitin/proteasome pathway. Upon activation, NRF2 protects cells from the consequences of oxidative stress, such as DNA damage and apoptosis (89, 139, 165). The NRF2-mediated cellular response to iron, including its cytoprotective role, has previously been demonstrated by our group. NRF2 regulates the response of hepatocytes against acute iron toxicity (153), prevents the development of liver fibrosis in iron-overloaded Hfe-KO mice (45), and regulates endothelial BMP6 secretion in response to iron overload (103).

The role of the NRF2-iron interaction in cell intrinsic and extrinsic (*i.e.*, niche) regulation of HSCs, however, remains to be explored. Nevertheless, several studies reveal the antioxidant transcription factor NRF2 as a significant player in hematopoiesis and in endothelial cells. Kim *et al.* showed that Nrf2 loss impairs the ability of hematopoietic stem and progenitor cell (HSPC) to repopulate, after BM transplantation (92). $Nrf2^{-/-}$ HSPC are functionally compromised, possibly due to impairment of the antioxidant defenses (92, 166). Furthermore, $Nrf2^{-/-}$ mice are more susceptible to radiation-induced cell damage after lethal irradiation (92). Keap1-KO mice, characterized by constitutive activation of Nrf2, have unaffected numbers of overall long-term HSCs (121). However, persistent activation of Nrf2 promotes cell cycle entry and further differentiation of these cells, leading to cell exhaustion and impairment of both HSC quiescent state and self-renewal capacity (121).

These results further demonstrate the importance of NRF2 regulation in maintaining HSCs. By having an impact on the differentiation of HSPCs, NRF2 activation enhances the granulocyte/monocyte lineage, suppressing erythroid differentiation (120). Studies conducted using $Nrf2^{-/-}$ mice showed that Nrf2-deficient HSCs are more susceptible to oxidative stress and that their function could not be retrieved with *N*-acetyl cysteine, suggesting that increased ROS alone are not responsible for defective HSC function in Nrf2-KO mice (114). On the contrary, Nrf2 activation increases HSPC function and mitigates irradiation-induced BM damage (92, 166). NRF2 has also been described as a key player in some hematological diseases, such as sickle cell disease (SCD). In a recent study, Keleku-Lukwete *et al.* used Keap1-KO mice to show that activation of Nrf2 results in a milder SCD phenotype, possibly due to downregulation of heme-driven cell activation (88).

Steady-State Erythropoiesis

Erythropoiesis is a highly regulated process, ensuring a constant supply of RBCs and is the largest consumer of iron in the body. Humans produce 1 million new RBCs per second throughout their life. RBCs arise from HSCs residing in the BM. HSCs differentiate into burst-forming units, which differentiate into colony forming units (CFUs) that trigger subsequent stages of differentiation (63). Formation of proerythroblasts marks the initiation of the third stage. The proerythroblast (or pronormoblast) divides to sequentially form the basophilic normoblast, polychromatic normoblast, orthochromatic normoblast, and the reticulocyte followed by enucleation and membrane remodeling (58). The later stages of differentiation take place in the circulation where reticulocytes undergo further maturation and are ultimately converted to mature RBCs (62). These steps are illustrated in Figure 4.

There are special niches in the BM known as erythroblastic islands that facilitate the enucleation and maturation of erythroblasts. Typically, an erythroblastic island comprises a central macrophage surrounded by erythroblasts (16, 35). A recent study has revealed expression of EPOR in central macrophages, further implicating their role in supporting erythropoiesis (102). Apart from supporting normal erythropoiesis, macrophages also play a role in regulating erythroid differentiation and proliferation in pathological conditions. Interestingly, preclinical studies have shown that treatment with clodronate, a first-generation bisphosphonate that has also been used to treat osteoporosis, can deplete macrophages and reverse the diseased phenotype of β-thalassemia and polycythemia vera (PV) (138). Moreover, macrophages also play a predominant role in recycling iron by scavenging senescent erythrocytes and hence support iron homeostasis (51).

Master regulators of erythropoiesis

The kidney is an efficient oxygen sensor, and under conditions of hypoxia, it produces EPO (25, 76). Binding of EPO to its cognate hypoxic-inducible receptor EPOR initiates an intercellular signaling cascade, culminating in the onset of the first phase of erythropoiesis. The EPO–EPOR interaction activates Janus kinase 2 (JAK2), a cytoplasmic tyrosine kinase and its downstream substrates, STAT5 and STAT3,

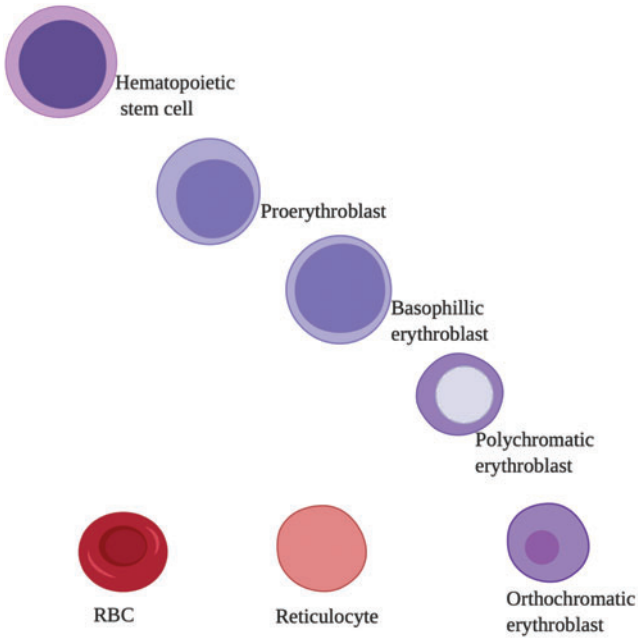

FIG. 4. Different stages of erythropoiesis. Erythropoiesis starts with the formation of hematopoietic stem cells and differentiates into proerythroblasts, which differentiate into basophilic, polychromatic, and orthochromatic followed by enucleation, resulting in the formation of reticulocytes and ultimately the RBCs. Color images are available online.

phosphoinositide 3-kinases (PI3K), and mitogen-activated protein kinase (MAPK), that promote the differentiation and proliferation of erythroid precursors (25, 141). Another erythroid transcription factor that regulates the expression of EPOR is GATA1 (64, 68). Gata1-KO mice die at E10.5–11.5 due to severe anemia. The conditional erythroid KO of this gene shows symptoms of aplastic anemia.

Apart from regulating heme synthesis, GATA1 also promotes early-stage proliferation and maturation by promoting the activation of survival genes such as BCL2 and BCL-xL. GATA1 is highly expressed at the CFU stage and is known to induce EPOR gene expression and its downstream partners MAPK/PI3K, which in turn phosphorylate GATA1 (68). Matured erythroid cells developing in an EPO-independent environment activate Fas death receptors in immature erythroid precursors, leading to caspase 3-mediated cleavage of GATA1 and apoptosis (68). In contrast, in early stages of erythropoiesis during EPO-dependent conditions, GATA1 is protected from caspase-mediated cleavage by the chaperone protein HSP70 to promote early erythroid maturation and differentiation. Apart from regulating steady-state erythropoiesis, GATA1 is also shown to be downregulated in several hematological disorders, such as β-thalassemia, MDS, and Diamond–Blackfan anemia. Inflammatory cytokines have been implicated in downregulating GATA1 expression, resulting in ineffective erythropoiesis (68).

Iron and Platelets

Clinically, iron deficiency is often accompanied by anemia as previously discussed. However, an intriguing common observation is the increased number of platelets, or thrombocytosis in patients with iron deficiency anemia. It has been

suggested that megakaryocytes are stimulated by EPO, but the mechanism remained elusive until recently. Xavier-Ferrucio *et al.* elegantly demonstrated that iron deficiency directly affects megakaryocytic erythroid progenitors (MEPs). The authors showed that low microenvironment iron content reduces ERK signaling and cycling of bipotent MEPs, modifies their metabolism, and biases their commitment toward megakaryocytes (172).

Iron overload and ROS were also shown to affect platelet function (40), as illustrated by hemochromatosis patients who present low thrombin-induced platelet aggregation (135). MDS patients with iron overload also have platelet dysfunction and an increased risk of hemorrhage. Increased iron levels have been correlated with the inhibition of γ-thrombin-induced platelet aggregation, through direct-binding effects (108). Popov *et al.* (135) described a correlation between ROS levels, leukopenia, and the degree of anemia. Moreover, ROS production led to a decreased platelet aggregation function, and therefore, to impaired platelet function as observed in patients with MDS (135). Furthermore, iron chelation therapy in MDS patients was able to recover platelet numbers in 78% of treated patients (80).

The recent discovery that platelets express proteins involved in iron metabolism, namely HFE and TFR2, suggested that platelets sense Tf saturation and play a role in iron metabolism (11, 69). Interestingly, Barale *et al.* (11) described that patients with iron overload have reduced response to aggregation stimuli and decreased surface expression of activation markers. Moreover, through *ex vivo* evaluation of platelet activation, they suggest that Tf saturation is inversely correlated with platelet reactivity (11).

Diseases That Arise from Dysregulated Erythropoiesis and Hematopoiesis and Novel Therapeutics for Restoring Iron Metabolism

HFE hemochromatosis

Hemochromatosis is characterized by dysregulation of systemic iron homeostasis. It can result from mutations in HFE (type 1) (OMIM #235200) (46) or mutations in other genes such as HAMP (type 2b) and HJV (type 2a) (OMIM #602390), FPN (SLC40A1) (type 4) and TFR2 (type 3) (OMIM #604250) (21) (26, 49, 93, 99, 136). Hemochromatosis is characterized by an excess of plasma NTBI, or labile iron (22). This excess iron leads to tissue toxicity and maximum iron retention in the liver, heart, and macrophages resulting in severe diseases such as hepatic carcinoma, cirrhosis, arrhythmia, cardiac failure, and diabetes (21). Increase in cellular iron stores can also hamper mitochondrial function (168). Current treatments include phlebotomy and the use of iron chelators such as deferasirox and desferrioxamine to manage iron levels (83, 130, 133). However, both treatments can result in attenuation of hepcidin, thereby increasing the rate of iron uptake. Moreover, iron chelators have other side effects and can impair auditory, renal, and ocular functions (94).

To mitigate these risks, several preclinical studies are currently testing the use of small interfering antisense oligonucleotides or siRNAs to target TMPRSS6 activity (2, 30). Casu *et al.* showed that the use of compounds targeting TMPRSS6 led to a reduction in hepatic iron content and an improvement of the iron-overload phenotype in a mouse model of β-thalassemia (30). Recent attention has also focused on the use of polyphenols as a dietary supplement, which can inhibit iron absorption by forming insoluble complexes with ferrous iron and hence are able to mitigate iron overload (https://clinicaltrials.gov/ct2/show/NCT03990181).

Furthermore, other preclinical discoveries such as mini-hepcidins/PR65 are shown to be effective in hepcidin KO models (137). Recent studies have also shown that thiazolidinones mitigate iron overload in hemochromatosis models by inducing hepcidin expression through activation of SMAD1/5/8 signaling (105). Targeting of the NTBI transporter ZIP14 in hemochromatosis mouse models was also shown to reduce iron overload in the liver and pancreas (78).

β-*Thalassemia*

β-Thalassemia is an autosomal recessive disorder that results from a mutation in the β globin gene of the hemoglobin molecule (142, 143). Consequently, there is increased production of α chains leading to an imbalance between the proportion of α and β chains. This leads to the formation of hemichromes, which damage RBC membranes and shorten their life span (142, 161). There are two types of thalassemia: (i) thalassemia major (TM) also known as transfusion-dependent thalassemia and (ii) thalassemia intermedia (TI) also known as nontransfusion-dependent thalassemia (161). TM patients require blood transfusions and iron chelation therapies (126). On the contrary, patients with TI do not require regular transfusions at an early stage, but develop anemia at a later stage and eventually require transfusions. A common manifestation of the disease is an increased rate of iron absorption that results in iron overload and thereby leads to the overproduction of ROS and oxidative stress. This causes cardiac siderosis, liver cirrhosis, osteoporosis, and skeletal deformities, as illustrated in Figure 5.

In β-thalassemia, excessive generation of ROS leads to apoptosis of a subset of polychromatophilic erythroid cells (112), thereby leading to ineffective erythropoiesis. On the contrary, an excess of α globins also binds to HSP70. HSP70 is known to protect GATA1, the major erythroid transcription factor. As a result, GATA1 is more prone to cleavage by caspase-3, which hampers erythroid maturation and differentiation and leads to inefficient erythropoiesis. Other studies have also shown that HSP70 sequestration can lead to ineffective erythropoiesis in β-thalassemia (6).

Current treatments of β-thalassemia include blood transfusions, hydroxyurea, iron chelators (DFO and deferasirox), HSCT, and splenectomy. Unfortunately, these treatments are often associated with severe adverse effects (94). To specifically target ineffective erythropoiesis, recent studies explored ACE-536, also known as Reblozyl/luspatercept-aamt, in thalassemia. ACE-536/luspatercept is a fusion protein with a modified extracellular domain of activin receptor IIB (ACVR2B) that competes with ACVR2B to bind members of the transforming growth factor (TGF) β superfamily (146) and is now a Food and Drug Administration-approved treatment for β-thalassemia (https://www.fda.gov/drugs/drug-approvals-and-databases/drug-trials-snapshots-reblozyl). Originally, luspatercept was thought to function as a trap ligand that binds growth differentiation factor 11 (GDF11), a member of the TGF-β family (159). It was therefore originally considered that luspatercept prevented the negative effect of TGF-β superfamily signaling in erythropoiesis.

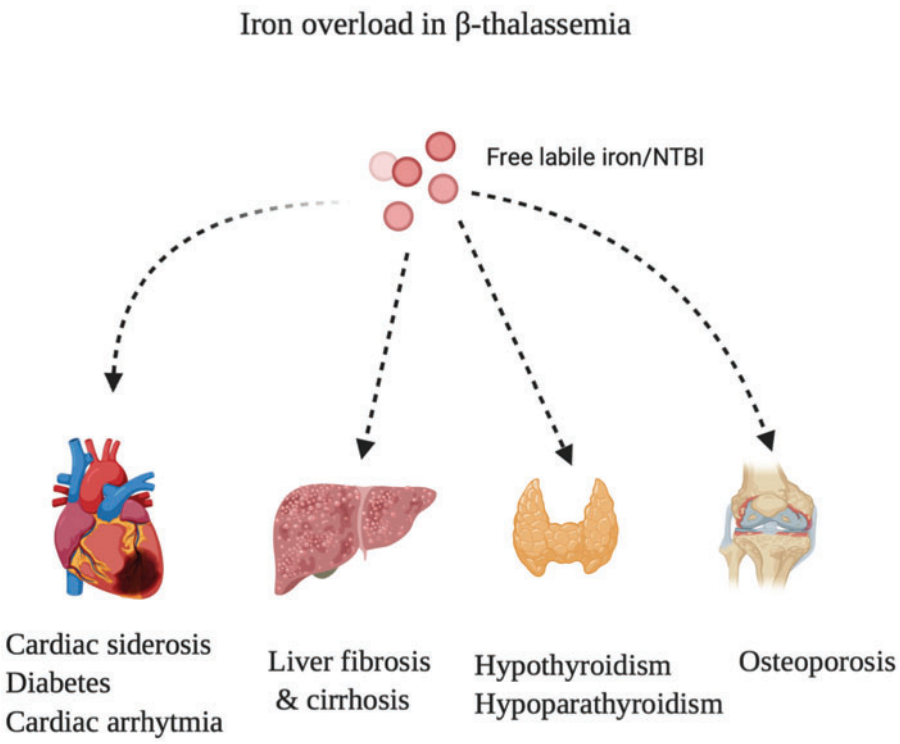

FIG. 5. Organs affected by iron overload. An illustration showing the different diseases that are caused due to iron overload in the liver, heart, bone, and thyroid glands. Color images are available online.

However, in a recent study using genetic mouse models, Guerra *et al.* showed that animals do not show improved recovery from erythropoiesis by conditionally deleting Gdf11 in a mouse model of thalassemia (65). Furthermore, the authors challenged Gdf11-deficient mice with the murine analogue of ACE-536 (RAP-536) and observed an improvement in hematological parameters of thalassemic mice (65). Altogether, this supported the notion that the GDF11 pathway is not underlying the benefit of ACE-536 in β-thalassemia. This work has been further supported by evidence that GDF11 does not play a significant role in regulating hematopoiesis (60). Moreover, recent studies propose that ACE-536 restores GATA1 expression in erythroid precursors and transcription intermediary factor 1γ (TIFγ) in the erythroblasts of β-thalassemic mice (111).

Other promising therapies are also being explored in β-thalassemia. Tf therapy was shown to be effective in preclinical studies, as the mice showed improved erythropoiesis and anemia (101). Additional studies have shown that the JAK2 inhibitor ruxolitinib can reverse splenomegaly in β-thalassemic mouse models and a Phase II clinical trial has been initiated based on these results (32). Although β-thalassemic patients receiving ruxolitinib showed a reduction of spleen size, overall, there were no changes observed in serum iron, ferritin levels, or pretransfusion hemoglobin levels (160). Due to these reasons, a Phase III trial with ruxolitinib was not initiated. Preclinical studies also explored the use of the FPN inhibitor VIT-2763 (140). VIT-2763 has been shown to ameliorate oxidative stress, improve erythropoiesis by reducing the aggregation of α chains hence mitigating iron overload in thalassemic mice (110). Iron chelators combined with amlodipine, an L-type calcium channel blocker, were also used in a recent randomized clinical trial in pediatric patients (90).

Minihepcidins were also tested in preclinical studies to reduce iron overload in β-thalassemic mouse models (31, 65). Based on these observations, two Phase II clinical trials were started to test LJPC-401 and PTG-300. Other modulators of hepcidin have been also shown to be effective in preclinical studies in treating β-thalassemia. Using antisense nucleotides and lipid nanoparticle siRNAs targeting TMPRSS6, researchers were able to mitigate iron overload and improve ineffective erythropoiesis in β-thalassemic mouse models (30, 56, 67, 150). Moreover, several new additional compounds targeting TMPRSS6 have been identified (14). A clinical trial will be also starting soon based on these results. Recent clinical trials are shown in Table 1.

Lastly, studies have also shown that monoclonal antibodies targeting ERFE are efficient in reducing serum iron levels and decreasing splenomegaly in thalassemic mouse models. ERFE has been known to reduce hepcidin expression upon stimulation by EPO and this therapeutic intervention will be extremely beneficial in treating iron-overload disorders (4). The different treatment approaches are illustrated in Figure 6.

Table 1. Interventions and Undergoing Clinical Trials for β-Thalassemia

Intervention	*Stage*
Reblozyl (luspatercept-aamt)	FDA approved
VIT 2763 (ferroportin inhibitor)	Phase I
LJPC-401 (minihepcidin)	Phase II—NCT03381833
PTG-300	Phase II—NCT03802201
IONIS TMPRSS6-LRx/ TMPRR6 inhibitors	Phase II—NCT04059406

FDA, Food and Drug Administration.

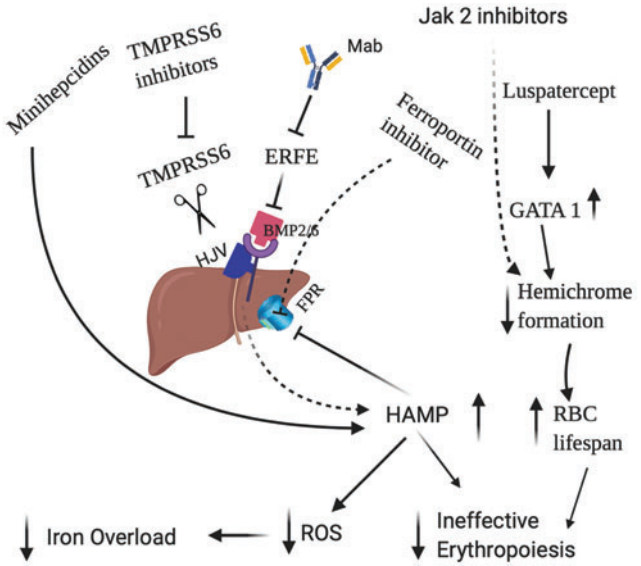

FIG. 6. Different therapies that are known to mitigate the iron overload and ineffective erythropoiesis in β-thalassemia. Iron restriction is perturbed by different hepcidin agonists, inhibitor of ferroportin, the use of TMPRSS6 inhibitors and monoclonal antibodies targeting ERFE, whereas improvement of erythropoiesis is modulated by luspatercept and JAK2 inhibitors by increasing the differentiation of erythroid progenitors and decreasing the formation of hemichromes. JAK2, Janus kinase 2. Color images are available online.

Anemia of inflammation

In several pathological conditions such as aging, hematologic and solid malignancies, autoimmune disorders, and chronic infections, normal erythropoiesis is severely compromised and myelopoiesis and lymphopoiesis are stimulated due to host-defense mechanisms (52). This condition is known as AI or anemia of chronic disease, which is characterized by an inflammatory state and chronically elevated cytokine levels. In the immediate innate phase, cytokines including IL-6 and tumor necrosis factor upregulate the transcription factor PU.1 and downregulate GATA1, thereby inhibiting the proliferation of erythroid precursors, which results in anemia. Administration of iron is not beneficial as it contributes to the generation of ROS and oxidative stress, as shown in patients with chronic kidney disease (39). In addition, inflammation also blunts the BM responsiveness to EPO (118).

IL-6 is known to be one of the predominant cytokines causing AI. It stimulates hepcidin, the master regulator of iron homeostasis, thereby leading to hypoferremia and limiting microbial proliferation. Consistently, patients with anemia of chronic disease have increased serum IL-6 and hepcidin levels (177). The hepcidin-induced decrease in iron bioavailability is a major contributor to anemia. Symptoms include dyspnea, fatigue, exercise intolerance, and headache. Patients with underlying health conditions such as diabetes and rheumatoid arthritis are more often anemic and symptomatic. Current therapies in AI include erythropoietic stimulating agents, RBC transfusions, and intravenous iron injections. As discussed, administration of iron may be detrimental in this setting, particularly in patients who are more susceptible to oxidative stress such as patients with cardiovascular disease. Iron may also increase the rate of infections. Several compounds under investigation for the treatment of AI are listed in Table 2.

Polycythemia vera

PV is a myeloproliferative neoplasm that is mainly due to JAK2 V617F activating mutations. The common symptoms are fatigue, pruritus, night sweats, bone pain, and weight loss. Overactivation of the JAK2 signaling pathway leads to constitutive activation of the downstream pathways, leading to uncontrolled proliferation and hematopoietic output. Ongoing preclinical and clinical studies include the use of pegylated interferon alfa-2a (156), use of the JAK2 inhibitor ruxolitinib, pegylated interferon alfa-2a (Phase II) (155, 176), and of the HDAC inhibitor or givinostat (Phase II Clinical trial: NCT03287245) and minihepcidins (31) (https://clinicaltrials.gov/ct2/show/NCT04057040).

MDS and leukemia

MDS is a heterogeneous group of clonal hematological diseases characterized by impaired hematopoietic differentiation and cytopenias, including anemia, with different

Table 2. Anemia of Inflammation Interventions, Targets, and Clinical Trials

Intervention	*Target*	*Status*
Tocilizumab (Mab)	IL6-receptor inhibitor	Phase II (72)
Etanercept, adalimumab anf Infliximab	TNF-alpha receptor antagonists	Phase II (38)
Heparin	Hepcidin antagonist	Preclinical (134)
Hemojuvelin-Fc, LDN193189	BMP antagonist	Preclinical (164)
Spiegelmer lexaptepid pegol	Hepcidin inhibitor	Phase 1 (19)
PRS-080-PEG30	Hepcidin antagonist	Preclinical (70)
LY2928057/Mab	Targeting ferroportin	Phase 1 (152)
LY3113593/Mab	Targeting BMP6	Phase 1 (152)
LY2787106/Mab	Targeting HAMP	Preclinical (147) Phase 1 (167)
Monoclonal antibody	Targeting hemojuvelin	Preclinical (97)
Momelotinib	ACVR1/Jak inhibitor	Preclinical (8) Phase III (115)
FG-4592	Prolyl hydroxylase inhibitor	Preclinical (12) Phase II (37)

BMP, bone morphogenetic protein; IL-6, interleukin 6; TNF, tumor necrosis factor.

degrees of severity (84, 131). The risk of MDS patients to progress to secondary AML can be stratified based on hematological and karyotypic features. Anemia is a major problem in MDS patients and the traditional approach relies on repeated RBC transfusions. Iron overload is a common side effect of RBC transfusions (151). Interestingly, patients with MDS with ring sideroblast have increased systemic iron concentration even before receiving RBC transfusions. These patients are characterized by ineffective erythropoiesis and mutations in the splicing gene SF3B1. A recent study elegantly demonstrated that SF3B1-mutated erythroblasts express higher levels of ERFE, which in turn suppresses hepcidin and leads to iron accumulation (17).

Iron overload in MDS is associated with comorbidities and increased mortality (151). Oxidative stress is linked to atherosclerosis, and iron overload is a risk factor for cardiovascular disease. Transfusion-dependent MDS patients are twice as susceptible to infection-related complications (41). Indeed, increased ferritin levels promote the proliferation, survival, and evolution of fungi, viruses, and bacteria (96, 117). In addition, cellular iron amount and phagocytic activity are inversely correlated (23). Monocytes have impaired activity against bacteria, and $CD4^+$ T lymphocytes have decreased proliferation and activity upon iron overload (148, 170). Transfusion-dependent MDS patients are also more likely to transform to AML, according to retrospective studies. (33). Altogether, these observations support the use of iron chelators in low- or intermediate-low-risk MDS patients, which is also thought to protect nonmalignant hematopoiesis from iron overload and reduce cytopenias (163).

Ferroptosis and malignant hematopoiesis

In 2012, Dixon *et al.* described ferroptosis, a new form of cell death induced by erastin that is morphologically, biochemically, and genetically different from apoptosis, necrosis, necroptosis, and autophagy (42) (Fig. 7). Ferroptosis is characterized by lipid peroxidation and may result from toxic accumulation of iron, leading to an increase in lipid ROS levels. Although other enzymes may also play a role, ROS are fundamental in ferroptosis, as indicated by the absence of ferroptosis induction under anoxic conditions (42). Ferroptosis has been associated with several conditions, including ischemic heart disease, kidney injury, and cancer,

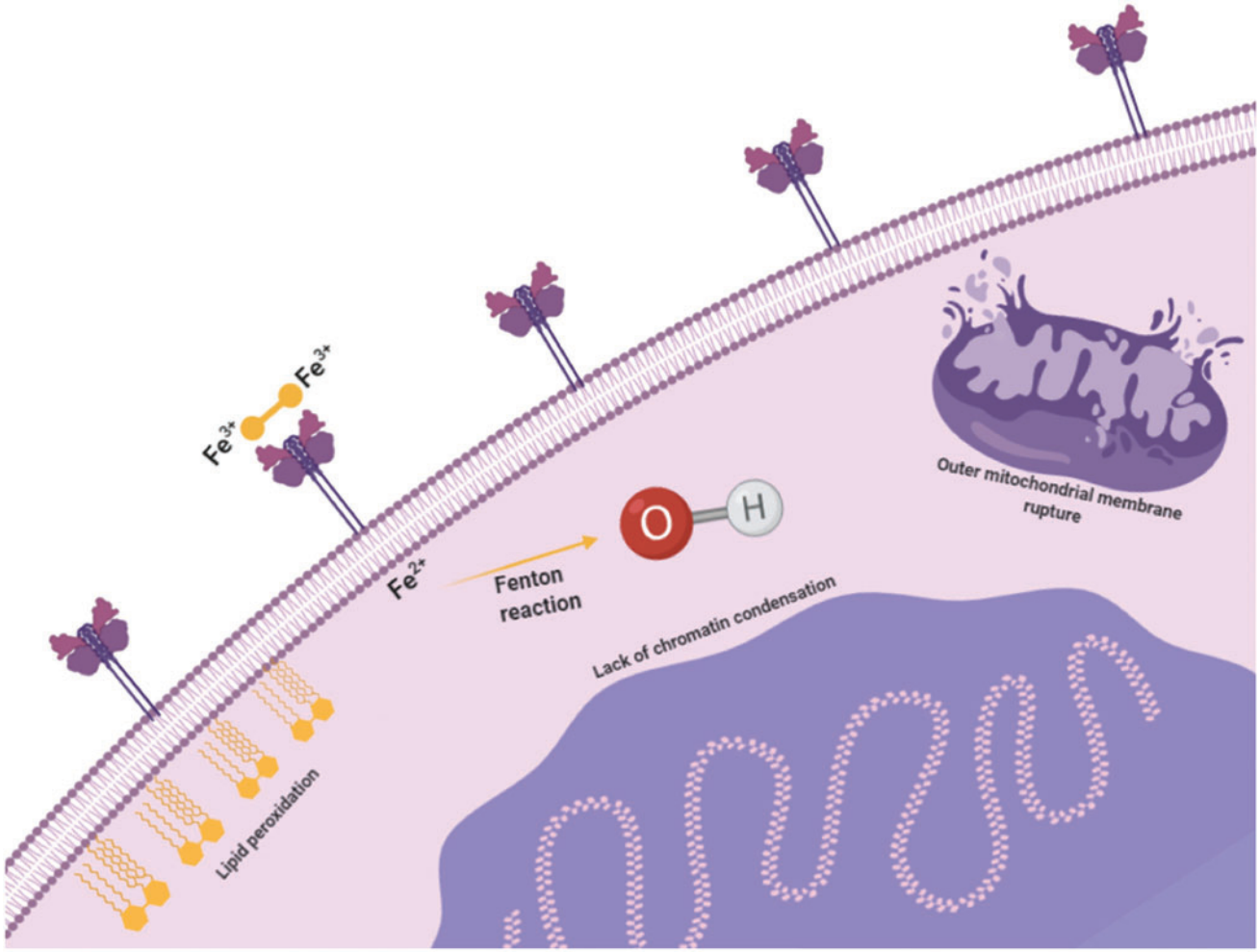

FIG. 7. The ferroptotic process. Increased ROS caused by iron toxicity trigger the onset of ferroptosis, leading to the activation of MAPKs and NAPDH oxidation. Several morphological features distinguish ferroptosis from other types of cell death, including mitochondrial membrane rupture, the lack of blebbing of the plasma membrane, and absence of chromatin condensation. MAPK, mitogen-activated protein kinase. Color images are available online.

in which it has been shown to promote tumoral growth through inactivation of p53 activity (55, 174).

At a morphological level, ferroptosis is characterized by the rupture of the outer mitochondria membrane, lack of nuclear chromatin condensation, and absence of plasma membrane blebbing. Aside from the previously mentioned iron and ROS accumulation, ferroptosis is also characterized by the activation of MAPKs, glutathione (GSH) depletion, and increased NAPDH oxidation, also creating a proinflammatory niche through the release of damage-associated molecular patterns (174). Furthermore, the importance of iron metabolism in ferroptosis is highlighted by the sensitization of erastin-induced cell death after silencing of FBXL5, which ubiquitinates and negatively controls IREB2/IRP2, a master regulator of several iron transport and storage genes that recognizes iron-responsive elements (42, 179). Even though excessive iron can mediate ferroptosis through the generation of ROS, other sources can increase the intracellular oxidative stress with the same outcome, such as NADPH-dependent lipid peroxidation and GSH depletion.

Yang *et al.* evaluated the effect of erastin-triggered ferroptosis in several suspension cell lines, including diffuse large B cell lymphoma (DLBCL), AML, and multiple myeloma (MM) cells. DLBCL cell lines were more susceptible to ferroptotic cell death, which could be rescued by antioxidants, such as vitamin E (178). Susceptibility to iron toxicity is being tested as a potential adjuvant therapy for MM. MM is a malignant clonal condition characterized by the uncontrolled proliferation of plasma cells (PCs) in the BM, accompanied by high levels of circulating immunoglobulins and organ damage (27). PCs contain high levels of hydrogen peroxide thus making them constitutively vulnerable to iron toxicity. Bordini *et al.* observed that in the Vk*MYC mouse model of MM, the combination of iron with bortezomib resulted in a synergistic effect and over 80% of the treated mice had partial or complete response and no disease progression (18).

Campanella *et al.* described similar results, demonstrating that the concomitant treatment with bortezomib and iron resulted in accelerated cell death also through apoptosis (27). In another study (181), tested the susceptibility of AML cells to chemotherapy (cytarabine and doxorubicin) in combination with erastin. They found that erastin was able to enhance the efficacy of the treatment, through the induction of ferroptosis (181). Altogether, ferroptosis inducers may be useful as adjuvant therapy in different hematological malignancies.

Conclusions

Iron homeostasis is maintained by a complex systemic regulation of iron intake, absorption, and recycling. Dysregulation in these pathways may result in iron deficiency or overload. Experimental and observational evidence demonstrates the importance of iron in the regulation of normal and malignant hematopoiesis. Many studies have explored the key role of iron in erythropoiesis. Several promising new therapies have emerged from these studies and are currently in use or under clinical trials for conditions such as SCD, AI, or PV. Future studies addressing the active (and not just the bystander) role of iron in HSC maintenance, niche homeostasis, and in hematological malignancies such as AML and MDS are welcomed.

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Author Disclosure Statement

S.R. is a member of the scientific advisory board of Ionis Pharmaceuticals, MeiraGTx, and Disc Medicine and owns stock options from Disc Medicine and MeiraGTx. S.R. has been or is a consultant for Cambridge Healthcare Research, Celgene Corporation, First Manhattan Co., FORMA Therapeutics, Incyte Corp, Ghost Tree Capital, Keros Therapeutics, Inc., Noble insight, Protagonist Therapeutics, Sanofi Aventis U.S., Inc., Slingshot Insight, Techspert.io and venBio Select LLC, and Disc Medicine.

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Abbreviations Used

ACVR2B = activin receptor IIB
AI = anemia of inflammation
AML = acute myeloid leukemia
BM = bone marrow
BMP = bone morphogenetic protein
CFU = colony forming unit
DFO = deferoxamine
DLBCL = diffuse large B cell lymphoma
E = embryonic day
EPO = erythropoietin
EPOR = erythropoietin receptor
ERFE = erythroferrone
FBXL5 = F-box and leucine-rich repeat protein 5
FDA = Food and Drug Administration
FLVCR1 = feline leukemia virus, subgroup C, receptor 1
FPN = ferroportin
GDF11 = growth differentiation factor 11
HAMP = hepcidin gene
HFE = hemochromatosis-associated protein
HJV = hemojuvelin
HSC = hematopoietic stem cell
HSCT = hematopoietic stem cell transplantation
IL-6 = interleukin 6
IRP2 = iron regulatory protein 2
JAK2 = Janus kinase 2
KEAP1 = Kelch-like-ECH-associated protein 1
LCI = labile cellular iron
MAPK = mitogen-activated protein kinase
MDS = myelodysplastic syndrome
MEP = megakaryocytic erythroid progenitors
MM = multiple myeloma
NCOA4 = nuclear receptor coactivator 4
Nfe2l2 or Nrf2 = nuclear factor (erythroid-derived 2)-like 2
NTBI = nontransferrin bound iron
PC = plasma cell
PI3K = phosphoinositide 3-kinases
PV = polycythemia vera
RBC = red blood cell
ROS = reactive oxygen species
SCD = sickle cell disease
STAT = signal transducer and activator of transcription
Tf = transferrin
TFR1 = transferrin receptor 1
TFR2 = transferrin receptor 2
TGF = transforming growth factor
TI = thalassemia intermedia
TIFγ = transcription intermediary factor 1γ
TM = thalassemia major
TMPRSS6 = matriptase-2
TNF = tumor necrosis factor