Cellular iron regulation in animals: need and use of suitable models

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Abstract

As with virtually all biologically essential transition metals, but probably in a more acute way than most, iron excess and deficiency underlie a range of pathological conditions in animals. Accordingly, regulatory systems maintain the proper iron amount to fulfill the needs of the whole body and of each individual cell, while avoiding deleterious effects. The latter may be due to lack of iron availability, e.g. at the active site of iron enzymes, or to reductive catalysis promoted by uncontrolled ferrous ions leading to the formation of reactive species such as the hydroxyl radical. Two major regulators maintain metazoan iron homeostasis, a systemic one relying on the circulating hormone *hepcidin*, and a ubiquitous cellular one organized around the *Iron Regulatory Proteins*. These central nodes of iron homeostasis are themselves regulated by numerous effectors beyond iron availability, and they impact other biological processes not directly

connected to the use of iron by animal cells. Further, the use of iron resources and conditions impacting it, such as variations of the redox balance, regulate cell fate, e.g. self-renewal of stem cells and differentiation in hematopoiesis. Iron and redox homeostasis are grounded on a series of identified molecular events, but it is not clear how changes of the associated biological parameters may favor proliferation of leukemic clones detrimental to maturation, in acute myeloid leukemia for instance. It now appears that the complex interactions among the networks influencing iron and redox homeostasis should be treated with new integrated data and modeling tools, with the aim to provide a global view of the functional differences between normal and pathological hematopoiesis in particular. The outcomes of the currently on-going efforts in this area are presented herein.

Introduction

The supply of essential trace elements to living cells needs to be secured for ensuring mandatory biological functions, and it has to be regulated to avoid unwanted effects. The latter statement is particularly important for iron which can easily catalyze highly deleterious biochemical reactions when not properly directed to its targets, generally the active site of various enzymes and proteins [1, 2].

Recent observations have indicated that cellular iron handling is coupled to other major biological functions, such as oxygen management [3], in shaping phenotypes. Basic biological functions at different levels (cellular, whole organisms) are set by series of regulatory and metabolic reactions involving iron and oxygen. They define networks and are interconnected. Hematopoiesis, and its deregulation in myeloid leukemia, is used herein to illustrate the importance of the iron and redox balances, and to stress the

usefulness of powerful new modeling approaches in analyzing complex biological processes.

Overview of iron homeostasis

Iron exchanges throughout the body.

In mammals, iron is provided by the diet and it is absorbed in the enterocytes of the proximal intestine [4]. Upon reaching the circulation by export through ferroportin, also called MTP1 or IREG1 (SLC40A1), iron is bound to transferrin and cells usually receive it by endocytosis of the transferrin-transferrin receptor complex. Most of the circulating iron is targeted to the bone marrow to be inserted into hemoglobin which concentrates 70-80% of the iron needs for oxygen distribution and carbon dioxide removal. Iron is recovered from senescent red blood cells in macrophages and re-injected into the circulation [5].

Liver is used as a storage organ in case of excess but no dedicated excretion system for body iron is available. Losses only occur by bleeding and cell peeling. Reciprocally, intestinal iron absorption is a relatively slow process that cannot be rapidly enhanced by several orders of magnitude. This explains the difficulties in recovering from nutritional iron deficiency, a major cause of morbidity worldwide. Thus, the limited exchanges of iron between animal bodies and their environment justify the presence of strict regulatory systems monitoring the biological use of the metal.

Systemic iron regulation

A general control mechanism is carried out by hepcidin, a 25 aminoacid hormone which is mainly synthesized in the liver and which interacts with ferroportin to trigger its degradation [6]. This mainly decreases iron absorption and recycling. The regulation of hepcidin is complex. Transcription of the gene responds to *i)* iron availability, *ii)* iron needs, and *iii)* inflammation. Hepcidin deregulation triggers diseases of iron

homeostasis, such as most types of hemochromatosis (iron overload) and iron refractory iron-deficiency anemia [7].

Cellular iron regulation

Beyond regulation by hepcidin, each cell has to adjust its iron provision and use to its needs. To this aim, the Iron Regulatory Proteins (IRP) bind to the Iron Responsive Elements (IRE) of regulated mRNA in cases of iron shortage [1]. Such binding increases – for the transferrin receptor- or decreases – for the ferritin subunits, ferroportin, and other messengers-translation (Figure 1). The two known IRP are mainly regulated at the post-translational level.

Overview of the cellular redox balance

Oxygen participates to fulfill the energy requirements of animal cells at the level of the mitochondrial respiratory chain, and other biochemical reactions catalyzed by dioxygenases also require oxygen as substrate.

Oxygen reduction and oxidative stress

Upon reduction of oxygen, intermediates such as the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical, may form [8]. They can also be generated by enzymatic reactions, as part of innate immunity against foreign substances for instance. Some derivatives of nitrogen monoxide share with partially reduced oxygen species a high ability to react with cellular components. The cellular equilibrium between reducing (i.e. electron donating) and oxidizing (i.e. electron withdrawing) molecules is referred to as the reduction-oxidation, i.e. *redox*, potential. Imbalance toward oxidation defines oxidative stress which, when not buffered by reducing compounds and activities leads to irreversible damage of cell components (e.g. proteins, nucleic acids, lipids, etc.).

Redox signaling via oxygen and its derivatives.

Conditions of even minor oxidative stress or the activity of specific enzymes may modify components of regulatory pathways and shift the set of networks cells rely on to function [9]. These changes impact cellular fate, be it programmed death, proliferation, or differentiation.
Examples of signaling molecules responding to redox shifts include transcription factors, e.g. of the nuclear respiratory factor (Nrf) family or activator protein 1 (AP-1), and signal transduction cascades, involving MAPK (mitogen-activated protein kinases) and protein tyrosine phosphatases. DNA oxidation may increase and induce repair by use of apurinic/apyrimidinic endonucleases, such as Apex1 (Ref-1), which are sensitive to redox changes.

The known roles of iron and of the redox balance in hematopoiesis

Hematopoiesis and acute myeloid leukemia.

Hematopoiesis is the biological process producing all blood cells. It mainly occurs in the bone marrow (of adults) and follows a succession of cell transformations from a limited number of stem cells. The generic term acute myeloid leukemia (AML) clusters conditions characterized by the proliferation of immature myeloid clones detrimental to the homeostasis of the bone marrow and, later, to the production of mature blood cells [10]. In general, the disease is of poor prognosis whatever the stage at which hematopoiesis is impaired; the latter varies, as reflected in the myriad of somatic mutations identified in AML clones. The drugs being presently used to treat AML aim at decreasing DNA replication, considering that malignant cells more heavily rely on this process as compared to non-cancerous ones [11, 12]. This strategy cannot cure the disease, but it is valuable to prepare patients for transplants. However, a single (or a limited

number of) drug targeting a general and essential feature of all AML clones would be economically and, hopefully, scientifically efficient, but this requires a more thorough understanding of the disease than presently achieved.

Redox control of myeloid differentiation

The many cellular crossroads that occur throughout hematopoiesis are paralleled by the modulation of many regulatory circuits. Throughout, the cellular redox balance play a role, with changes activating pathways or occurring through the action of leukemic oncogenes in hematopoietic malignancies [13-15]. For instance, two of the frequently mutated genes in AML, fms-related tyrosine kinase 3 (Flt3) with internal tandem repeat (ITD) and Ras, appear to increase the redox potential of hematopoietic stem cells [16].

The general physiological status with respect to oxygen can also impact hematopoiesis. Hypoxia triggers erythropoietin synthesis by the activation of hypoxia induced transcription factors (HIF) [17]. This activates erythroid precursors and enhances production of red blood cells, hence iron consumption. In contrast, the buildup of oxidative species in the bone marrow activates FoxO transcription factors [18] which regulate a range of genes involved in the antioxidant response, DNA repair, and cellular fate. FoxO are inhibited by Akt serine threonine kinase which transduces signals from inositol 1,4,5-trisphosphate (PI3)-kinase. The latter responds to different stimuli such as growth factors and stress effectors.

Hematopoietic stem cells gather in the most hypoxic regions of the bone marrow [19, 20], and it seems that differentiation follows the oxygen gradient in the bone marrow and, beyond, into the circulation. Leukemic blasts endure high levels of oxygen without maturating, thus showing

deficiency to detect, respond, and integrate the variations of the redox potential accompanying hematopoiesis.

Roles of iron in the cell cycle: relevance to myeloid differentiation

Because unregulated transition metals, and iron prominent among them, display a large catalytic potential to interconvert oxygen-bearing molecules in the cellular context, the link between redox regulation of hematopoiesis and iron handling is expected to be very tight. Proliferating cells exposed to iron chelators rapidly stop growing and die mainly by apoptosis [21]. An early identified target of chelators is the ribonucleotide reductase R2 subunit which requires iron to generate the tyrosyl radical initiating de-hydroxylation of ribonucleotides and electrons from thioredoxin or glutaredoxins. This enzyme is one of the points of convergence between cellular iron management and redox regulation. In addition cyclins A, E, and D, some dependent kinases such as CDK2 and CDK4, and of CDK inhibitors, such as $p16^{INK4}$, $p21^{WAF1/CIP1}$, or $p27^{KIP1}$ are iron regulated, and they interact with major regulators of the cell cycle, such as the retinoblastoma protein (pRb) and p53. Modulation by iron seems to occur at the transcriptional, translational, or post-translational levels.

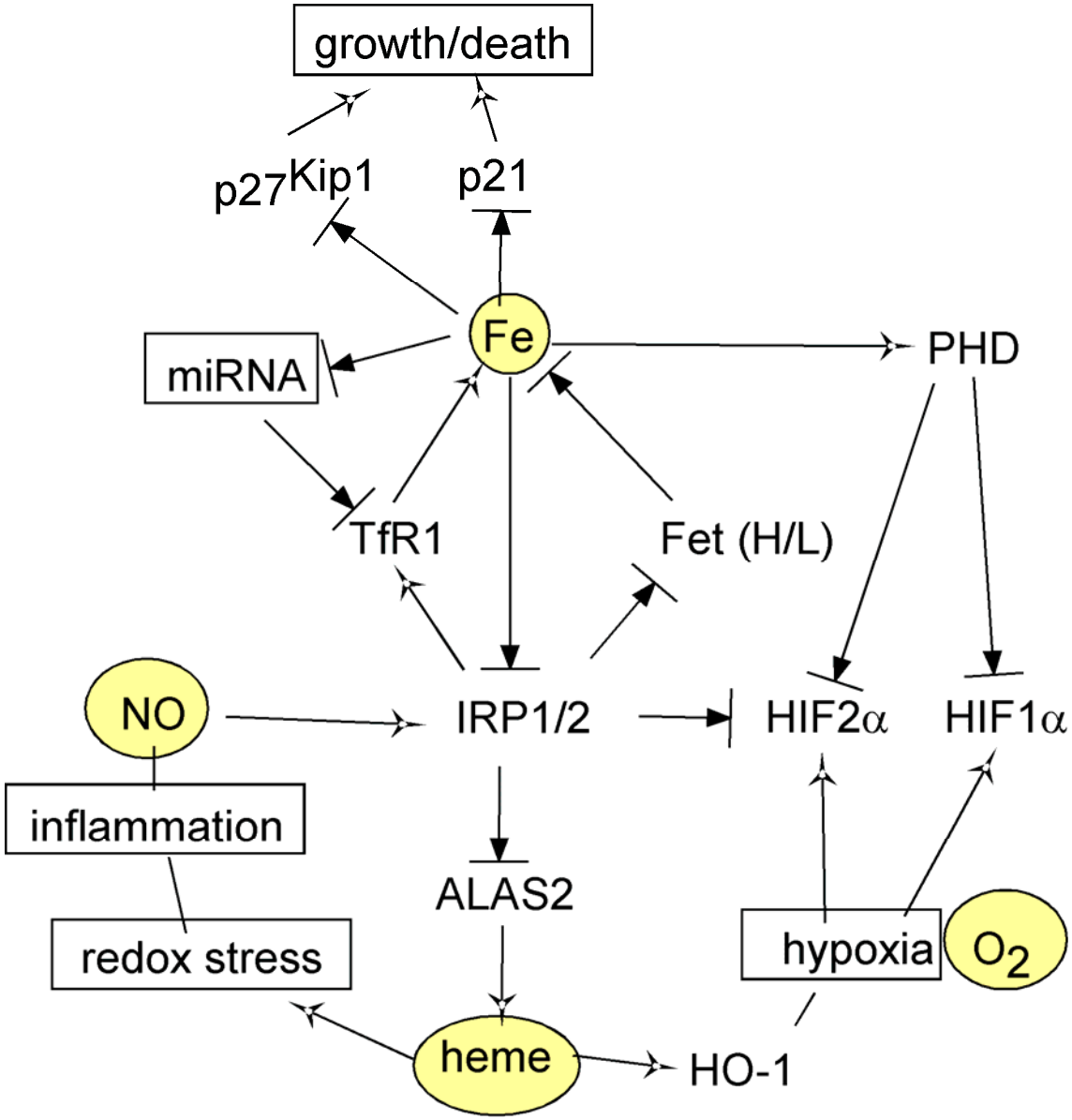
Of particular interest, the regulation of N-myc downregulated gene 1 (NDRG1) occurs through hypoxia, NO, N-myc as the name says, and other regulators and it may upregulate $p21^{WAF1/CIP1}$. Exposing cells to iron chelators increases NDRG1, and, in AML samples, NDRG1 levels seem lower than in different myeloid cells from normal donors, thus associating NDRG1 up-regulation and differentiation [22]. Accordingly, NDRG1 expression increases during erythropoiesis [23].

Perturbation of the cell cycle by iron chelation may involve other pathways. Treatment of the human erythroleukemia cells K562 by deferasirox (ICL670, Exjade®) acts on mTor (mammalian target of

rapamycin) [24], an important modulator of cell death and proliferation which is also a downstream target of Akt and PI3K. More generally, suppression of the iron provision to leukemic cells [25, 26] revealed commitment into the monocyte lineage and increased apoptosis, with initial, over a few hours, increased levels of oxidative species and activation of mitogen-activated protein kinases (MAPK).

At another regulatory level, micro-RNA modulate expression of genes involved in iron homeostasis, either upstream or in parallel of the hepcidin and IRP driven systems [27]. In the case of myeloid leukemic cells, the involvement of mi-RNA in the commitment into different lineages antagonistic to proliferation has been highlighted: the identified mi-RNA target the transferrin-receptor and the non IRE-regulated DMT1 transcripts [28, 29]. Reciprocally, iron homeostasis influences processing of precursors into mature mi-RNA [30]. Further, iron influence on major cellular functions can be extended to post-translational processes, such as protein modification, e.g. [31], or degradation, e.g. [32].

From the above, it should appear that iron and redox homeostasis interact at many stages. A scheme recapitulating some of the involved pathways is shown in Figure 1. Myeloid maturation is a relevant context for these interactions by its sensitivity to available iron and to redox imbalance.



Legend to Figure 1. Schematic view of some interactions occurring among participants to iron and redox homeostasis in animal cells, particularly hematopoietic ones, as discussed in the text. Small molecules triggering effects are highlighted in yellow. Blocked arrows indicate inhibition. HO: heme oxygenase; ALAS2: erythroid aminolevulinate synthase; other abbreviations are given in the text.

The use of modeling iron homeostasis in hematopoiesis

To illustrate the complexity of AML patho-physiology, single mutations of the CCAAT/enhancer-binding protein alpha (CEBPA) gene in a fraction of the heterogeneous family of cytogenetically normal AML [33] lead to a worse overall survival of the patients than double, mainly biallelic, ones [34]. This counter-intuitive outcome suggests that the activity of CEPBA contributes to worsen AML prognosis, whereas its inhibition proportionally improves it. However, the mechanism explaining why a regulatory module, which is sensitive to iron and redox homeostasis most likely through the HIF pathway, should be knocked-down to repress proliferation of AML progenitors is not straightforward. This highlights the remaining gaps in basic human biology and in pathological mechanisms to predict, or even describe, the pathological outcome with obvious consequences for patient care. Modern modeling approaches may provide means to deal with such problems.

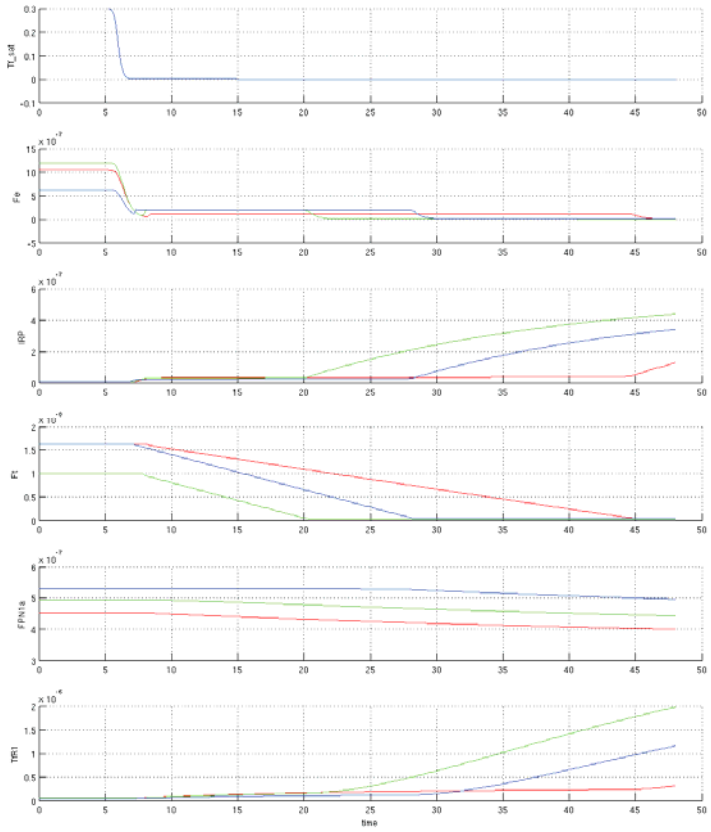
Some implemented methods to model iron homeostasis

The considered question of cellular iron homeostasis, redox balance, and hematopoiesis calls for the integration of various biological data. Thus modeling should involve systems described at various levels, but no actual multi-scalar approaches have yet been applied in this field.

The latest kinematic model of animal iron homeostasis adjusted kinetic constants and other parameters describing the exchanges between the different pools of iron in the mouse body and their evolution upon changes of the nutritional iron provision [35]. A conceptually different approach considered an abstracted and digitized representation of iron homeostasis at the level of the whole body using the language of Petri nets [36]. This modeling process can analyze physiological (e.g. impact of hepcidin production) and pathological (e.g. anemia associated with inflammation) conditions by modulating the transitions represented in the model [37-39].

In a further step toward a formal and digitized representation of iron homeostasis, models implementing boolean networks, in which each element can be in one of two states, may be proposed. This has been used with the available biochemical information on iron handling in the well characterized eukaryote *Saccharomyces cerevisiae*. The Boolean formalism was extended by adding a weight, representing the probability of occurrence, to each reaction [40]. The result is a comprehensive description of more than 10^3 biochemical reactions among more than 600 elements involving, directly or indirectly, iron in yeast. Known phenotypes, such as growth with different substrates or after removal of selective genes in mutants, or upon applying a physiological switch, e.g. +/- O_2, were analyzed for this unicellular organism under laboratory conditions.
The cellular level studied in yeast is also relevant for biological events which are initiated and develop locally in a single tissue, such as the growth of a leukemic clone in AML for instance. An early attempt at considering mammalian cellular iron homeostasis with switch-like regulatory steps, i.e. by representing steep transitions between regulated and non-regulated states, integrated data available at the time [41]. The dynamic properties of this system were represented by ordinary differential equations (ODE). A more recent model of this kind included the presence of the cellular iron exporter, ferroportin, and its degradation upon interaction with external hepcidin [42]. This modeling approach was set in the context of breast cancer, and it particularly focused on properties that were independent of the values of the model parameters. A qualitative validation of this model was provided by modulating ferroportin production, and measuring variations in the concentrations of ferritin and IRP(2).

Another option for the same system has been recently proposed [43]. It differs by the differential equations used to describe the system, particularly in the analytical form representing regulation by available iron and IRP. Indeed, this theoretical discrepancy cannot yet be resolved by discriminatory experimental data. However, the latest efforts [43] *i)* could restrict the set of parameters relevant to a stable physiological condition, *ii)* they could monitor the dynamics of the system upon perturbing this condition by relying on the behavioral constraints expressed in a temporal logic formalism, and *iii)* they bore significant predictive power as to the time evolution of the system triggered by environmental changes. Figure 2 shows the outcome of these simulations. The molecular consequences newly revealed by these results will be experimentally probed in the future, thus significantly extending the present knowledge on this important regulatory system.



Legend to Figure 2.

Conclusion

Therefore, the latest developments in the modeling of the core regulatory network managing iron at the cellular level provide insight into the details of its properties. These improvements are now challenging existing experimental data and they call for new measurements. This to-ing and fro-ing between experiments and modeling should further detail the placement of this regulatory system among the set of networks organizing mammalian cells.

When combining a robust molecular description of iron regulation with that of redox balance within the already well advanced modeling of

hematopoiesis [44, 45], it may be hoped that a more integrated view of pathological myeloid differentiation will emerge and will improve the therapeutic strategies currently implemented to treat AML.

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