MOLECULAR COORDINATION OF

IRON HOMEOSTASIS BY MICRORNA

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HOMEOSTASIS BY MICRORNA

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Abstract: Iron is an essential nutrient critical for oxygen transport, DNA synthesis, ATP generation, and cellular proliferation. At the molecular level, insufficient iron elicits a cascade of cellular events aimed at conserving iron for the maintenance of these life-preserving functions, but tissue-specific responses and metabolic adaptations to iron deficiency (ID) are not fully understood. Recently, small regulatory RNA molecules called microRNA (or miRNA) have been identified as an important mechanism for regulating various cellular processes. Therefore we sought to determine if the expression pattern of miRNA changes in response to dietary ID and to examine the potential regulatory capacity of miRNA in the adaptive response to ID. To do this, we first characterized the expression of miRNA in the livers of iron-sufficient and iron-deficient animals using next-generation sequencing technology. Results compiled from three different bioinformatics approaches indicate that ~10 miRNA are differentially expressed in the livers of ID rats. Further bioinformatics analyses suggested that at least two of these miRNA, miR-210 and miR-181d, had predicted targets directly involved in either the maintenance of iron homeostasis or the metabolic adaptation to iron deficiency. We then used reporter assays to validate the putative miRNA targets including the miR-210 target, cytoglobin, and the miR-181d targets, carnitine palmitoyltransferase 1B and mitoferrin 1. These findings have provided insight into the metabolic adaptation to ID and have demonstrated how miRNA contribute to the molecular coordination of iron homeostasis in a physiologic model of dietary ID.

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CHAPTER I

INTRODUCTION

Iron is an essential, yet potentially toxic nutrient; thus iron homeostasis must be tightly regulated to ensure adequacy and prevent overload. Iron balance is maintained by two regulatory systems that function to coordinate iron homeostasis at both the systemic and cellular levels. While the machinery controlling these two systems is different, there is considerable overlap in the molecular components that each of these systems control, and as such both must function synergistically to coordinate vertebrate iron metabolism. Yet, many questions remain as to how these systems communicate with one another, particularly in situations where iron availability is altered and iron homeostasis is disrupted.

Central to the potential for iron to accumulate and promote cell damage through the production of free radicals is the absence of a regulated mechanism to promote iron efflux from the body. Systemic iron homeostasis is tightly maintained through the regulation of intestinal iron absorption and recycling of iron from specialized cells that are components of the reticuloendothelial system (RES). The key iron regulatory peptide

hormone hepcidin is primarily responsible for coordinating systemic iron homeostasis by affecting the rate of intestinal absorption and/or iron release from cells of the RES [1]. When hepatic iron stores are elevated, hepcidin expression, synthesis, and secretion is increased to regulate systemic iron metabolism. Hepcidin represses cellular iron export by binding to the iron export protein ferroportin (Fpn) and promotes Fpn internalization, ubiquitination, and subsequent proteasomal degradation [1, 2]. Thus, with elevated iron stores, iron is retained within enterocytes and the iron-recycling macrophages of the RES thereby limiting iron absorption and release from iron stores. Conversely, when iron stores are low, hepcidin expression is suppressed and intestinal iron absorption and iron release from the macrophages of the RES is enhanced in an effort to restore iron homeostasis [2].

While hepcidin is generally considered to be the primary means of regulating systemic iron homeostasis, a family of cytosolic RNA binding proteins known as Iron Regulatory Proteins (IRP) is considered to be the global regulators of *cellular* iron homeostasis. IRP regulate cellular iron homeostasis by "sensing" intracellular iron status and coordinating the uptake, storage, and utilization of iron accordingly. The two members of this family of RNA binding proteins include IRP1 and IRP2, both of which coordinate cellular iron homeostasis through high-affinity binding to stem-loop structures known as Iron Responsive Elements (IRE) in either the 5' or 3' untranslated region (UTR) of mRNA encoding proteins involved in iron metabolism. These highly conserved stem-loop structures are present in mRNA encoding proteins of iron uptake (transferrin receptor 1, or TFRc and divalent metal transporter 1, or DMT1), iron storage (heavy or H- and light or L-ferritin), iron utilization (mitochonodrial aconitase or, ACO2), and iron export (ferroportin, or FPN) [3]. When cytosolic iron levels are limiting, IRP bind to IRE with high affinity thereby

inhibiting the translation of mRNA containing 5' IRE, such as ferritin, or stabilizing mRNA containing 3'IRE, such as TFRC [3]. Conversely, with elevated cytosolic iron levels, IRP lose their high-affinity RNA binding activity resulting in the de-repression of ferritin synthesis and degradation of TFRC mRNA [3, 4].

While IRP1 and IRP2 both possess the same RNA binding function, they are regulated through distinct iron-dependent mechanisms. IRP1 is a bifunctional protein that can exhibit either high-affinity RNA binding activity (apoIRP1) or enzymatic activity by functioning as the cytosolic isoform of the tricarboxylic acid cycle enzyme aconitase (c-acon or holoIRP1) [3, 4]. Under iron-replete conditions, the formation of a [4Fe-4S] iron-sulfur cluster is favored and is associated with a reduction in high-affinity RNA binding activity and subsequent increase in enzymatic activity of c-acon [5]. However, when iron is limiting, formation of the Fe-S cluster is impaired (or the cluster is disassembled) and holoIRP1 is converted to its active high-affinity RNA binding form (apoIRP1) [3-5].

Despite an approximate 61% amino acid identity and 79% amino acid similarity, IRP2 lacks the cysteine residues required to coordinate a [4Fe-4S] iron-sulfur cluster and therefore exhibits no enzymatic activity as an aconitase [6]. Another important difference between IRP1 and IRP2 is the insertion of a 73 amino acid sequence in IRP2 that contributes to the iron- and oxygen-dependent modulation of protein stability and degradation [7]. Under iron-replete conditions, IRP2 is targeted for proteasomal degradation via an iron-, oxygen-, and 2-oxoglutarate-dependent prolyl hydroxylase and subsequent recognition by the E3-ubiquitin ligase F-box and leucine-rich repeat protein 5 (FBXL5) [8]. Under iron-deficient conditions IRP2 is stabilized by inhibition of the prolyl hydroxylase and de-stabilization of FBXL5 [9].

Interestingly, the effects of iron deficiency (ID) on IRP function and activity appear to be tissue-specific. For example, in the livers of ID animals, IRP1 binding activity only increases to about 10% of the available pool of IRP1 protein, suggesting that even under ID conditions ~90% of the protein exists as c-acon/holoIRP (Clarke and Eisenstein, unpublished data). However, in skeletal muscle (i.e., gastrocnemius and soleus), nearly 100% of the available IRP1 protein pool is converted to apoIRP1 under iron-deficient conditions (Clarke and Eisenstein, unpublished data). Further, the total abundance of IRP1 is decreased in skeletal muscle, but not liver, in response to ID. These findings not only provide evidence of a tissue-specific response, but also reveal an additional mechanism (via decreased protein stability, enhanced turnover, repressed translation, or decreased mRNA abundance) that may play a role in controlling IRP1 activity. Indeed, as described above, the primary means of regulating IRP1 activity is through the formation or loss of a [4Fe-4s] iron-sulfur cluster. Previous studies have also demonstrated significant reductions in mitochondrial enzyme activity, Fe-S cluster enzyme content, and the Fe-S cluster assembly cysteine desulfurase protein (IscS) abundance in skeletal muscle in response to dietary ID [10-12]. In fact, the majority of the negative health consequences resulting from iron deficiency arise from alterations in iron metabolism in skeletal muscle [13]. This observation has largely been attributed to impaired Fe-S cluster protein function that are essential for numerous biologic processes including maintenance of iron homeostasis, mitochondrial respiration, electron transfer, cellular metabolism, and gene regulation [13, 14].

The indispensable nature of iron is also reflected by its critical role in many cellular processes including oxygen transport, DNA synthesis, ATP generation, and cellular differentiation and proliferation. Unfortunately, ID remains a major public health concern,

affecting as much as 25% of the world's population [15]. ID progresses in stages and can occur with and without anemia. Anemia occurs in the final stage of ID when iron depletion is severe and there is an inadequate supply of iron to support erythropoiesis. Symptoms of ID anemia include weakness, fatigue, reduced capacity to transport oxygen, impaired cognitive function in children, and a reduced ability to fight infection [16, 17].

In animal models, less well characterized responses to ID include alterations in lipid and glucose metabolism as a result of decreased oxidative capacity, leading to a shift in preferential fuel utilization from fat to glucose [18-20]. These animals also display signs of disrupted metabolic homeostasis as they exhibit hyperglycemia, hyperinsulinemia, and hyperlipidemia presumably as a result of alterations in insulin signaling [18, 21]. Another interesting finding in the investigation into the metabolic response to iron deficiency is that the severity of these consequences (i.e., hyperglycemia and hyperlipidemia) appears to be a graded response to a reduction in hemoglobin [22]. There is an inverse correlation between decreasing hemoglobin levels through the progression of anemia and elevated serum levels of lipids and glucose. The extent to which these metabolic responses associated with ID are the result of a physiologic adaptation to iron deficiency, or pathologic consequences of insufficient iron availability remains relatively unknown.

Regardless, a reduction in iron status is clearly associated with negative physiologic effects. In addition to the consequences associated with the loss of Fe-S cluster protein activity, a major reason for the adverse side effects observed with ID is due to the requirement of iron for the biosynthesis of heme, which as the primary component of hemoglobin makes oxygen transport possible. Additionally, other heme-containing proteins (e.g., cytochromes) have critical roles in ATP generation, lipid metabolism, and steroid

hormone synthesis [13, 23]. Iron in the form of heme also acts as a cofactor in regulating protein function. For instance, heme binding enhances the regulatory capacity of the transcriptional repressor Rev-erbα, and thereby implicates iron as having potential roles as a key integrator of circadian and metabolic pathways [24]. Recent findings have expanded the physiologic roles of heme even further as a potential regulator of mRNA stability and degradation via the critical role it has been shown to play in microRNA (miRNA) processing [25, 26].

miRNA are a class of noncoding RNA approximately 22 nucleotides (nt) long that are predicted to regulate as much as 60% of all protein-coding genes, and thus contribute to the coordination of a variety of biological processes [27, 28]. miRNA are potential candidates for the currently unidentified tissue-specific regulation of IRP in response to ID as they are often expressed in tissue-specific patterns and may affect both the spatial and temporal regulation of many protein-coding genes [29, 30]. In fact, the oxygen sensitive miR-210 has been shown to regulate the protein abundance of iron-sulfur cluster assembly proteins (ISCU1/2) in cultured cells, and therefore could potentially play a significant role in the regulation of IRP1 activity as well [31, 32]. Also, the liver-specific miR-122 has been shown to play a significant role in the regulation of lipid metabolism and systemic iron homeostasis in mice, and likely contributes to the metabolic response to ID [33, 34].

As mentioned above, the negative health consequences resulting from ID are primarily due to alterations in iron metabolism in skeletal muscle [13, 35, 36]. Interestingly, the liver appears to be relatively resistant to the effects of ID in terms of mitochondrial metabolism and heme-containing protein functions, though relatively little is known about the molecular mechanisms regulating iron metabolism in different tissues and how alterations

in iron status in different tissues affect iron homeostasis. As mentioned above, one potential candidate regulatory mechanism is mediated by the iron-dependent expression of miRNA. Indeed, there is accumulating evidence suggesting that miRNA may also contribute to the coordination of mammalian iron homeostasis [37]. This evidence for the role of miRNA in modulating iron homeostasis is underscored by the fact that miRNA processing is, at least in part, a heme-dependent process [25, 26].

The rationale for the proposed work is that determination of the roles of miRNA in coordinating the molecular response to changes in iron status will provide fundamental insights into the understanding of how iron homeostasis is maintained and how alterations in iron sensing can lead to the development of disease. Thus, our primary objectives were to (1) examine miRNA expression profile under iron-adequate and iron-restricted conditions in animals, (2) to identify differentially expressed miRNA, (3) to examine the potential targets of differentially expressed miRNA, and (4) to characterize the impact of miRNA expression on putative targets involved in iron metabolism. The central hypothesis was that miRNA expression would be regulated in response to ID and that these changes would be associated with changes in the expression of target mRNA resulting in the homeostatic regulation of cellular iron metabolism. This research has provided insight as to how miRNA contribute to the metabolic adaptation to the iron deficiency and the molecular coordination of iron homeostasis.

CHAPTER II

REVIEW OF LITERATURE

Chalybeate (iron-containing) waters in Europe were first recognized for their healing properties in medieval times. Later, in the 1600s, iron fillings were steeped in wine, and supplemented orally to ward off chlorosis, a condition we now recognize as anemia [38]. This is quite remarkable as the role of iron in the development of anemia was not properly described until the 1930s [39, 40]. As a result of these findings, developed countries began fortifying flour and processed foods with iron in the 1940s in an attempt to stave off iron deficiency, a practice that still continues in the present day. Additionally, numerous pharmacological resources now exist for the treatment and correction of iron deficiency. Yet, despite the tremendous expansion in our understanding of the etiology of iron deficiency anemia, and massive treatment and prevention efforts, iron deficiency remains a major public health problem.

The persistence of iron deficiency throughout the millennia is multi-faceted. The unregulated, but nominal excretion of iron by humans is insufficient to deplete body iron stores, and thus iron sufficiency is largely regulated at the level of intake and absorption.

Nutritional iron deficiency occurs when dietary iron intake or iron absorption does not meet physiological requirements, and can result from several factors. For example, rapid growth coupled with a low iron diet can result in iron deficiency in children, and premenopausal women may become iron deficient due to frequent heavy menstrual blood loss. In under-developed countries, blood loss as a result of parasitic infection can also exceed dietary iron intake, resulting in iron deficiency [17]. Other conditions that often result in iron deficiency include infections, tumors, inflammation, and genetic disorders.

Iron deficiency is a major public health concern because a reduction in iron status can result in significant negative physiologic effects. Symptoms of iron deficiency include weakness, fatigue, reduced work capacity, impaired cognitive function in children, and increased susceptibility to infection [17]. Globally, iron deficiency affects billions of people and its symptoms are attributed to the loss of millions of dollars, and to the death and disability of more than 800,000 individuals annually through increased risk of child and mother mortality, reduced fitness and productivity, and cognitive impairment [41, 42]. Thus, understanding the pathology of the manifestations of iron deficiency is of utmost importance, because although the causes are clear, iron deficiency persists, and the consequences are significant. Outlined below is a detailed review on our current understanding of iron needs, metabolism, and regulation, and the exquisite molecular controls coordinating iron homeostasis.

The necessity and toxicity of iron

The maintenance of optimal iron status is critical for numerous reasons. Too little iron can result in the development of anemia while too much iron is toxic and can lead to

tissue damage and failure. The former is most often caused by dietary intake that is insufficient to meet physiologic needs, while the latter is most commonly caused by diseases of iron metabolism, such as hereditary iron overload. Remarkably, the features of iron that are attributed to the crux of its essentiality, the ability to undergo oxidation and reduction, are the same properties that largely contribute to its potential for toxicity. In addition to its properties as a transition metal however, iron is also an essential nutrient because of the vital role it plays in many life preserving functions including oxygen transport (hemoglobin), cellular respiration (cytochromes), and DNA synthesis (ribonucleotide reductase).

The daily production of hemoglobin containing red blood cells accounts for the majority of iron utilization in the body and represents nearly 80% of the iron demand in humans [43]. Each day the body produces approximately 200 billion new red blood cells, each of which contains millions of hemoglobin molecules, requiring some 20 mg of iron per day [43]. Hemoglobin is the primary oxygen transporter from the lungs to various tissues, and thus is essential for respiration. It is the presence of the iron atom at the center of heme that makes the transport of oxygen by hemoglobin possible. When insufficient iron is available for optimal hemoglobin synthesis, anemia ensues, total oxygen carrying capacity of the blood decreases, and the symptoms of iron deficiency begin to manifest [13].

Iron, in the form of heme, also has many other biologic functions. The presence of iron in heme-containing cytochromes enables the transport of electrons. In the electron transport chain, cytochromes, such as cytochromes b and c, pass along single electrons; the transfer of which is made possible by the change in the oxidation state of

iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state [44]. It is this oxidation of nutrients through the electron transport chain that makes their energy release to the body possible. Other heme-containing cytochromes, such as cytochrome P-450 are involved in oxidative degradation of drugs and steroid hormone synthesis [13]. Iron in the form of heme also acts as a cofactor in regulating protein function. For instance, heme regulatory motifs have been characterized in three major circadian regulators: neuronal PAS domain protein 2, nuclear receptor subfamily 1, and Period 2 [23]. Recent findings have expanded the physiologic roles of heme even further as a potential regulator of mRNA stability and degradation via its critical role in microRNA processing, a function which will be discussed in much more detail below [25, 26]. Thus, heme can serve as an integrator of mammalian energy metabolism and circadian rhythm as well as a regulator of mRNA expression and function.

Non-heme iron containing compounds also comprise an important group of proteins and enzymes essential for normal physiologic function. Proteins containing iron-sulfur (Fe-S) clusters are found in virtually all living cells and within multiple cellular compartments including the mitochondria, cytosol, and nucleus [45]. Fe-S clusters in proteins act as cofactors that are essential for numerous biologic processes including maintenance of iron homeostasis (IRP1), mitochondrial respiration (mitochondrial aconitase), electron transfer (NADH), and DNA repair (Fanconi anemia group J and *Xeroderma pimentosum* group D) [14, 46]. Expression and function of Fe-S proteins is largely influenced by alterations in iron status, and likewise mutations in Fe-S cluster assembly proteins are causative of human disease.

Despite the essentiality for iron in critical life preserving functions, it is equally important to note there is also considerable potential for iron toxicity. Because there is not a regulated mechanism to control iron excretion, excess iron can accumulate in body tissues and organs. As mentioned above, it is the redox capability of iron which contributes both to its essentiality and toxicity. In excess, this redox activity can lead to the generation of damaging free radicals via Fenton chemistry, which is the reaction between hydrogen peroxide and ferrous iron that can produce hydroxyl radicals and other oxidizing species capable of creating biological injury [47].

Indeed, iron accumulation in the brain has been linked to oxidative damage and neurodegeneration associated with multiple sclerosis, Friedrich's ataxia, Parkinson's, and Alzheimer's disease [48]. Additionally, oxidative stress and damage has been implicated as a causative factor for several chronic diseases including cardiovascular disease and diabetes [49, 50]. The role of iron in the pathogenesis of these two diseases has been supported by epidemiological evidence indicating that elevated iron storage levels are associated with increased risk of coronary heart disease and insulin resistance [50, 51]. Although an exact mechanism has yet to be elucidated, the potentially toxic characteristics of iron are suspected to play a critical role.

Unlike iron deficiency, iron overload and toxicity is rarely attributed to a dietary imbalance, and instead is most commonly observed in individuals with genetic conditions. For instance, hereditary hemochromatosis is the most common genetic iron overload disorder affecting approximately 1/200 individuals of northern European descent [52]. It is associated with mutations in the HFE gene and characterized by dysregulation of intestinal iron absorption and inappropriate parenchymal iron deposition

[53, 54]. Early symptoms of excess iron deposition include fatigue, joint pain, depression, impotence, and increased skin pigmentation [53]. Left untreated, patients with hemochromatosis accumulate iron in tissues like the liver, heart, and pancreas resulting in the development of cirrhosis, cardiomyopathy, and diabetes, respectively [53].

Several other iron overload or hemochromatosis disorders exist that are not associated with mutations in the HFE gene. For example, "juvenile or Type II hemochromatosis," is associated with the same phenotypic characteristics as type I hemochromatosis but is due to mutations in the gene encoding hemojuvelin (HJV) also known as HFE2 [43, 55]. Individuals with juvenile hemochromatosis accumulate iron at a much faster rate and tend to experience cardiomyopathy and other endocrinopathies rather than severe liver disease [55]. In the absence of treatment, these patients typically suffer from heart failure before age thirty [53, 55]. Other types of hemochromatosis can result from mutations in genes encoding the iron hormone hepcidin (HAMP1), the iron uptake protein transferrin receptor 2 (TfR2), and the iron export protein ferroportin [43]. The importance of each of these proteins in the regulation of mammalian iron metabolism is discussed in more detail below.

Iron absorption, transport, uptake, and storage

The control of iron homeostasis is primarily influenced by an individual's iron needs and status of body iron stores. Because there is no regulated means for iron excretion, homeostasis is primarily mediated through controlling dietary absorption. Intestinal iron absorption is largely influenced by organismal iron status, and may range from 10% (for an individual with normal iron status) up to 35% (for an individual who is

iron deficient) [56]. Iron absorption can occur throughout the entire length of the small intestine, but iron is most efficiently absorbed in the duodenum [56]. Dietary iron exists as either heme iron (from meats) or nonheme iron (from plants).

Heme iron is actually much more efficiently absorbed than nonheme iron, but the mechanisms by which it is absorbed remain poorly understood. Although heme transporters have been described, their roles in intestinal iron absorption are not fully agreed upon, and appear quite nominal [57-59]. However, it is generally believed that most dietary heme iron is internalized then disassembled by heme oxygenase to enter a common pathway with dietary nonheme iron. Most dietary nonheme iron is found in the ferric form, but must be reduced to ferrous iron either chemically or through the action of the iron-regulated brush border enzyme duodenal cytochrome reductase (Dcytb or Cybrd1) before being transported across the luminal membrane of the enterocyte by divalent metal transporter-1 (DMT1) [60]. The importance of Cybrd1 in non-heme iron absorption remains enigmatic however due to the finding that the loss of Cybrd1 has no effect body iron stores, indicating that alternative pathways for reduction of dietary iron likely exist in the intestine [61].

Regardless of form, once in the enterocyte, iron has three fates: (1) storage/excretion, (2) utilization, or (3) transport to other tissues. Iron that is not transported out of the enterocyte can be incorporated into the iron storage protein ferritin for short-term storage [62]. If iron is not needed, it can be "excreted" with the short-lived mucosal cells that are sloughed off every 2-3 days [63]. If needed however, iron can be released from ferritin for utilization by the intestinal cells as a cofactor for enzymes, or transported out for utilization by other tissues. Iron is transported out of the enterocyte

across the basolateral membrane by the iron export protein ferroportin (Fpn) [43]. Mutations in hemochromatosis gene product HFE result in an increase in the expression of DMT1 and Fpn in the duodenum contributing to the inappropriate increase in intestinal iron absorption and export in individuals with hereditary hemochromatosis [64].

Following export across the basolateral membrane, ferrous iron is oxidized by the multi-copper oxidase hephaestin (Heph) prior to being loaded onto the iron transport protein transferrin (Tf) [65]. Tf, a glycoprotein, binds a maximum of two iron atoms and serves as the primary means for interorgan transport [66]. Tf plays a critical role in iron transport as it has the capacity to reversibly bind iron. This is important because at physiological (neutral) pH, iron is insoluble in its free state and is capable of generating free radicals. However at a neutral pH, Tf binds to iron with high affinity making it safely available for transport to other body tissues such as the liver, muscles, and bone marrow [65].

Iron uptake in these tissues occurs through clathrin-dependent endocytosis of transferrin via interaction with transferrin receptors (TfR) 1 and 2 [62, 65]. Both TfR1 and 2 are capable of complexing with Tf for the internalization of iron, but while TfR1 is ubiquitously expressed, TfR 2 expression is limited to hepatocytes and erythroid cells [67]. TfR1 also has a much higher affinity for Tf than Tfr2, and as such represents the primary means of iron uptake in most cells [62, 67]. However, at least nine mutations have been characterized in TfR2 that lead to a severe early-onset form of hereditary hemochromatosis, and thus TfR2 is also a critical factor in the control of iron homeostasis [68]. Once bound, the Tf/TfR complex is endocytosed, where the acidic pH of the endosome results in the release of iron, and Tf and TfR are recycled back to the

cell surface where they disassociate upon encountering the nearly neutral pH [69]. The released iron is then reduced by Steap metalloreductases before being pumped into the cytoplasm, most likely by DMT1 [62, 69]. Once in the cytoplasm, iron is used by the cell (i.e., in the production of iron-containing proteins), exported out of the cell via ferroportin, or stored in the primary cellular iron storage protein, ferritin.

Ferritin is a cytosolic protein involved in iron storage and detoxification in microbial, plant, and animal species [62, 66]. In mammals, ferritin molecules are present as heteropolymers with 24 subunits of two types, H-subunits (heavy or heart) and L-subunits (light or liver) [70]. Both ferritin types are ubiquitously expressed, but their expression ratios vary greatly depending on the tissue and cell type, with H-ferritins predominating in the heart and brain, and L-ferritins being more prevalent in the liver and spleen [71]. H-subunits exhibit ferroxidase activity that promotes the loading of iron into storage, whereas L-subunits are more efficient at promoting mineralization of nuclei [72]. Homopolymers of H-type ferritin are also found in the mitochondria where they provide the same protective and storage functions as their cytosolic counterparts [71]. The delivery of non-utilized iron from the cytosolic pool to ferritin remains unclear, but the iron chaperone protein poly (C)-binding protein 1 (PCBP1) does facilitate the iron loading of ferritin *in vitro* and in cultured cells [71]. However, the contribution of PCBP1 to the loading of ferritin and the maintenance of iron homeostasis *in vivo* remains to be elucidated. Under physiologic conditions it is generally thought that iron release occurs with lysosomal or proteosomal degradation of ferritin, but it remains unclear how iron is then made available for use or transport [71].

The liver is a primary site of iron storage, accounting for approximately 60% of the ferritin in the body, but of particular significance is the remaining 40% of ferritin found in the cells of the reticuloendothelial system (RES) [73]. Iron is recycled by specialized macrophages within the RES that phagocytose senescent or damaged red blood cells and remove them from circulation [74]. This is important because while the 1-2 mg of iron absorbed each day is sufficient to counter obligatory iron losses (i.e., bleeding and sloughing off of mucosal and skin cells) of around 1 mg per day, daily erythrocyte production (200 billion red blood cells per day) requires 20-24 mg of iron for hemoglobin synthesis [43]. Thus, while absorption of dietary iron is important to satisfy daily iron losses, it is the body's mechanism of conserving and recycling iron that ensures proper iron stores.

Within the macrophages, red blood cells are lysed and hemoglobin is degraded by heme oxygenase which catalyzes the liberation of iron from heme [43]. Macrophages can then either store the iron derived from hemoglobin in ferritin or release iron through the iron export protein Fpn with the aid of soluble multi-copper oxidase cerruloplasmin [74]. The majority of iron entering the plasma for distribution or redistribution by transferrin is derived from the RES, sites of hemoglobin destruction, and/or ferritin and hemoglobin degradation [73]. Thus, cells of the RES and the signaling molecules that regulate their function play a critical role in maintaining whole-body iron homeostasis.

Systemic iron homeostasis

The adult human body contains iron in two major pools: 1) functional iron in hemoglobin, myoglobin, and enzymes and 2) storage iron in ferritin and transferrin. The majority of iron is found in the functional pool, with only about 20% remaining as

storage iron (found primarily in hepatocytes and the macrophages of the RES) [73]. Because of iron's essential, yet potentially toxic nature, iron homeostasis must be maintained at both systemic and cellular levels, and movement between these two pools must be tightly regulated. As there is no regulated mechanism for iron excretion, systemic iron balance is tightly maintained through the regulation of absorption from the intestine. Four situations lead to measurable changes in iron absorption: abnormal iron availability (overload or deficiency), accelerated erythropoiesis, hypoxia, and inflammation. [75, 76]. In this manner, iron absorption and plasma availability is decreased in response to iron overload and inflammation, and increased in response to an inadequate iron status, enhanced erythropoiesis, and hypoxia [43, 76].

Much enthusiasm was generated when the small peptide hormone hepcidin, initially thought to function as an antimicrobial agent, was shown to be a major regulator of both intestinal iron absorption and iron recycling within the RES [1, 2]. Hepcidin is now recognized as a key iron regulatory hormone responsible for coordinating iron absorption with existing iron stores to meet systemic iron needs. Hepcidin, which is secreted by the liver, regulates systemic iron metabolism by promoting the internalization, ubiquitination, and lysosomal degradation of the iron export protein Fpn in both enterocytes and the macrophages of the RES [1, 77]. Thus, when iron stores are elevated, hepatic hepcidin expression and secretion are increased and iron absorption and release from stores is diminished. Conversely, when iron stores are low, hepcidin expression is decreased and intestinal iron absorption and iron release from the RES is enhanced. Mutations in HAMP1, the gene encoding hepcidin, are associated with the

development of a severe type of juvenile hemochromatosis and illustrate the central role this peptide hormone plays in the regulation of systemic iron homeostasis [1, 2].

Regulation of hepatic hepcidin expression occurs at the transcriptional level. Hepcidin expression is decreased in response to situations such as anemia and hypoxia, but is increased in response to inflammation [76]. Key molecules in the regulation of hepcidin expression include the hemochromatosis gene product, HFE, the bone morphogenetic protein (BMP) co-receptor, hemojuvelin (HJV), and the iron sensor TfR2. One means of hepcidin regulation is through the binding of HFE with the iron uptake proteins TfR1 and TfR2. In this manner HFE has been suggested to act as a bimodal switch between these two iron sensors because high concentrations of transferrin bound iron displace HFE from TfR1 and promote its interaction with TfR2 [78]. The HFE-TfR2 complex then binds HJV and activates hepcidin transcription via BMP/SMAD signaling [78, 79].

While HFE and TfR2 interaction can contribute to hepcidin activation, hepcidin transcription is predominantly controlled through iron activated BMP6 interaction with the BMP co-receptor HJV. To date, how BMP6 mRNA expression is modulated in response to increasing and decreasing iron levels remains to be determined [62]. Once bound, the BMP-HJV complex activates hepcidin transcription by interacting with type I and II BMP receptors at the plasma membrane, which induces phosphorylation of receptor-activated SMAD (R-SMAD) proteins that can then dimerize with SMAD4 [80]. The R-SMAD/SMAD4 heterodimer can then translocate to the nucleus and activate transcription of the HAMP1 gene [77, 80]. Hepcidin regulation in response to erythropoietic signals is also influenced by BMP-SMAD signaling, which is inhibited by

the release of erythroid precursors [62]. Inflammatory stimuli such as interleukin 6 can also induce HAMP1 transcription through activation of STAT3 (which also requires the presence of SMAD4) and the subsequent binding of STAT3 to a regulatory element in the HAMP1 promoter [81].

Modulation of hepcidin expression in response to situations such as anemia, hypoxia, and inflammation suggest hepcidin is a key regulator of iron homeostasis under various pathophysiological conditions [76]. This evidence has been further supported by work demonstrating that complete lack of hepcidin in mice results in iron overload, while animals overexpressing hepcidin experience decreased body iron levels and severe anemia [82, 83]. Moreover, mutations in hepcidin regulatory molecules such as HFE, HJV, and TfR2 in humans result in iron overload due to the absence of hepcidin expression [84]. Similarly, mutations in the transmembrane protein matriptase 2 (TMPRSS6), which leads to inappropriately elevated hepcidin transcription, are the genetic basis for iron-refractory iron deficiency anemia [85]. Thus, an effective means of hepcidin administration in response to iron overload or a means to counteract the overexpression of hepcidin seen in genetic and inflammatory diseases could have momentous pharmacological value.

As described above hepcidin's primary mode of action for the maintenance of systemic iron homeostasis is through its posttranslational regulation of the iron exporter Fpn. Also mentioned was the fact that hepcidin gene expression is influenced by hypoxic stimuli, (which can enhance erythropoiesis), and alterations in iron levels (signaled by TfR). It is of note then that these three critical components of systemic iron homeostasis: cellular iron export (Fpn), iron utilization (erythropoiesis), and iron uptake (TfR), are

subject to tight regulation at cellular level as well. However, while the targets of systemic and cellular iron coordination overlap, the machinery controlling the cellular iron homeostasis is quite different.

Cellular iron homeostasis

The coordination of iron uptake, storage, and utilization is critical in maintaining optimal levels or iron and the appropriate distribution of the intracellular iron pool. A number of proteins intimately involved in the maintenance of cellular iron homeostasis are regulated post-transcriptionally by the so-called "global regulators" of iron metabolism, Iron Regulatory Proteins (IRP). It is through the actions of the two iron regulatory proteins, IRP1 and IRP2, which both act to "sense" the intracellular iron status, that total body iron homeostasis is tightly controlled. IRP regulate iron metabolism through high-affinity binding to highly conserved stem-loop structures of CAGUGX hexanucleotide loop sequence in mRNA termed Iron Responsive Elements (IRE) [4, 86]. These conserved stem-loop structures are located in either the 5' Untranslated Regions (UTR) or 3'UTR of mRNA encoding proteins of iron metabolism, and alter protein translation or mRNA stability, respectively [4].

Both IRP1 and IRP2 function as high-affinity cytosolic RNA binding proteins that are regulated in an iron-dependent manner. IRP1 is a bifunctional protein exhibiting either high affinity RNA binding protein activity or enzymatic activity by functioning as the cytosolic isoform of the TCA cycle enzyme aconitase (c-acon) [3]. The activity (or function) of the protein is largely dependent on the presence or absence of the iron-sulfur Fe-S cluster [3, 66]. Under iron replete conditions, the presence of a [4Fe-4S] iron-sulfur cluster confers enzymatic (aconitase) activity and inhibits high-affinity RNA binding

activity [87]. Conversely, under iron deficient conditions, the Fe-S cluster is "disassembled" resulting in the generation of high-affinity RNA binding activity (IRP1) [5].

In contrast to IRP1, IRP2 does not contain a [4Fe-4S] iron-sulfur cluster and lacks aconitase activity functioning only as an RNA binding protein. Rather than being regulated through the assembly or disassembly of an Fe-S cluster, IRP2 is regulated primarily through iron- and oxygen-dependent modulation of protein stability and degradation [6]. Aside from the lack of an Fe-S cluster, IRP2 also contains an additional 73 amino acid sequence that is necessary for its iron-dependent regulation and degradation [4]. Under iron-replete conditions, IRP2 is targeted for proteasomal degradation via an iron- and oxygen-dependent prolyl hyrodroxylase and subsequent recognition by the E3-ubiquitin ligase F-box and leucine-rich repeat protein 5 (FBXL5) [8, 88]. Under iron-deficient conditions IRP2 is stabilized by inhibition of the prolyl hydroxylase [3].

Various extracellular stimuli are capable of influencing IRP1/c-acon and IRP2 independent of cellular iron status. For example IRP1 activity is modulated in response to oxidative stress due to modifications of the [4Fe-4S] iron-sulfur cluster. Hydrogen peroxide, nitric oxide, and peroxynitrite are the most well-characterized cluster perturbants. They promote the loss or disassembly of the [4Fe-4S] iron-sulfur cluster generating the RNA binding form of the protein [4, 89]. Although interconversion of IRP1/c-acon via assembly and disassembly of the Fe-S cluster is thought to be the primary mechanism through which the protein's activity is regulated, IRP1 activity can also be regulated independently of iron by other means including oxidative stress and

post-translational modification [89, 90]. IRP1 is regulated by protein kinase C (PCK)-dependent phosphorylation at two PKC phosphorylation sites, S711 and S138 resulting in decreased aconitase activity and decreased Fe-S stability, respectively [91, 92]. IRP2 can also be regulated by phosphorylation [4]. Interestingly, phosphorylation of IRP2 was shown to increase RNA binding activity through the activation of a latent pool of IRP2 rather than an increase in protein synthesis [93]. Thus, IRP2 appears to be able to switch from a high-affinity phosphorylated RNA binding protein to a low-affinity dephosphorylated form through the regulation of phosphatases and protein kinases [4, 93].

Despite the multitude of differences in the regulation of IRP1 and IRP2, both exhibit similar genetic regulatory functions as central regulators of iron metabolism, regulating proteins involved in the uptake (DMT1), transport (TfR1), storage (ferritin), and utilization (erythroid aminolevulinic acid synthase) of iron. When cells are iron deficient, both IRP function as high-affinity RNA binding proteins and repress the translation of mRNAs containing IRE in their 5'UTR (i.e., ferritin) and increase the stability of mRNAs containing IRE in their 3'UTR (i.e., TfR1) [3]. Under iron replete conditions, IRP lose their high-affinity RNA binding capacity and fail to bind IRE thereby de-repressing ferritin synthesis and decreasing TfR mRNA stability [3].

In addition to regulating the expression of proteins directly involved in the maintenance of iron homeostasis, IRP also regulate mRNA encoding proteins involved in energy metabolism and oxygen sensing. For instance IRE have also been identified in the 5'UTR of mRNA encoding mitochondrial aconitase (m-acon) and the iron-protein subunit of succinate dehydrogenase (SDH - in Drosophila only), two TCA cycle

enzymes[4, 94]. The regulation of m-acon and SDH via the IRE/IRP system provides a direct link between iron and energy metabolism and is thought to play an important role in fuel utilization during iron deficiency [94, 95]. The discovery of an IRE in the 5'UTR of the hypoxic transcription factor, hypoxia inducible-2α mRNA provided new insight into the physiologic adaptation to iron deficiency [96]. It was well established for some time that the hypoxic down-regulation of hepcidin to increase iron availability for stimulation of red blood cell production served to meet physiological needs by increasing oxygen transport [76, 97, 98]. However, under iron limiting conditions, stimulation of erythropoiesis could lead to the production of hypochromic microcytic red blood cells due to impaired hemoglobin production and further deplete already low iron stores. The IRP-mediated functional repression of HIF-2α protein expression in response to iron deficiency shed new light onto how the rate of red blood cell production, and thus iron utilization, is adjusted based on iron availability [99, 100].

Interestingly, the effects of iron deficiency on IRP function and activity have been shown to be tissue-dependent. While the liver appears to be relatively resistance to changes in iron status, the skeletal muscle is severely affected [10, 13]. For instance, spontaneous binding activity of IRP1 in the livers of iron-deficient animals only increases to about 10% of the available pool of protein (Clarke, unpublished data). However, in skeletal muscle, nearly 100% of the available IRP1 protein pool is converted to the active IRE binding form under iron-deficient conditions (Clarke, unpublished data). As described above, the functional role of IRP1 is largely dependent on the presence of a [4Fe-4s] cluster. Intriguingly, the protein abundance of the mitochondrial cysteine desulfurase iron-sulfur cluster S (IscS) is negatively impacted in the skeletal muscle, but

not livers of rats fed an iron deficient diet [10]. Therefore, it is tempting to postulate the observed tissue-specific effects on IRP1 RNA binding activity are the result of iron-dependent effects on the Fe-S cluster machinery

Iron-sulfur cluster proteins

Fe-S clusters in proteins, such as IRP1, act as cofactors that are essential for numerous biologic processes including maintenance of iron homeostasis, mitochondrial respiration, electron transfer, metabolism, and many other regulatory processes [14]. Proteins containing Fe-S clusters are found in virtually all organisms, and within multiple cellular compartments including the mitochondria, cytosol, and nucleus. The synthesis and assembly of Fe-S clusters is a complex and highly regulated process involving the delivery of iron and sulfide to specific apoproteins located within the subcellular compartments [14]. A spectrum of human diseases associated with a dysregulation in cellular iron metabolism have been attributed to mutations in genes involved in Fe-S cluster biogenesis, such as the iron-sulfur assembly proteins Iscu1/2 [45, 46]. Given the importance of Fe-S proteins in the regulation of iron homeostasis (i.e., IRP1) and energy production (i.e., m-acon), which is reduced in iron deficiency, it is of interest to identify and elucidate regulatory factors involved in the formation and maintenance of Fe-S clusters, particularly in response to iron deficiency.

In mammals, more than ten proteins have been identified as having critical roles in the maturation of mitochondrial Fe-S proteins, although several more candidate proteins are predicted to be involved [45]. A key initial step in Fe-S protein biogenesis is the generation of sulfur by the cysteine desulfurase, nitrogen fixation homolog (Nfs1 or IscS) and its obligatory partner Isd11 [45]. The sulfane sulfur generated by Nfs1/Isd11

then has to be reduced, most likely by ferredoxin reductase and ferrodoxin, before being assembled onto the iron sulfur scaffold homolog, Iscu [46]. The movement of iron within the cell is one of the least understood problems in iron biology, so another initial, but less well defined step in Fe-S biogenesis is the transfer of iron onto the scaffold protein. However, two mitochondrial importers have been identified, mitoferrin 1 (Mfrn1 or Slc25a37) and mitoferrin 2 (Slc25a28), that can mediate the transport of iron in its reduced form into the mitochondrial matrix [45, 62]. The assembly of iron onto the scaffold protein is then believed to be mediated by the iron-binding protein frataxin by undergoing an iron-stimulated interaction with Nfs1/Isd11 [14]. Following assembly of a transient Fe-S cluster on Iscu, its transfer and assembly into apoproteins is facilitated by a mitochondrial monothiol glutaredoxin (GLRX5) and through coordination with specific amino acid ligands for formation of the final protein product [14].

As mentioned above, Fe-S cluster proteins are utilized in multiple subcellular compartments, but whether they all originate from the mitochondria is still up for debate. Nonetheless, and even though the molecular mechanisms remain poorly defined, maturation of both cytosolic and nuclear Fe-S proteins is absolutely dependent upon the function of the mitochondrial Fe-S assembly machinery [46, 62]. This is further evidenced by the requirement for the export of a yet unknown compound by the mitochondrial transporter ABCB7 for the maturation of cytosolic, but not mitochondrial Fe-S proteins [101, 102]. Thus, because mitochondria are the primary site of Fe-S cluster biogenesis (and heme synthesis), mitochondria represent a major subcellular site for iron utilization. It is important then that mitochondrial iron homeostasis be tightly regulated in an effort to preserve proper mitochondrial function. Recent work has shown that IRP

are critical for securing mitochondrial iron supplies and protecting against detrimental iron deficiency [103]. Interestingly, proper functioning of the mitochondrial Fe-S cluster assembly machinery itself is also required for mitochondrial iron homeostasis as depletion of Fe-S cluster biogenesis proteins results in marked iron accumulation [46]. Furthermore, mitochondrial iron overload is a feature of many human Fe-S cluster assembly disorders [46].

In addition to contributing to mitochondrial iron homeostasis, Fe-S cluster containing proteins have numerous other regulatory capacities. This is strongly evidenced by a number of diseases that are attributable to abnormal Fe-S cluster biogenesis, such as Friedreich's ataxia and X-linked sideroblastic anemia [45, 46]. However, it is of note that Fe-S proteins can also be affected by non-pathologic means as well. For instance, dietary iron deficiency can have a significant negative impact on Fe-S cluster protein abundance and function. Intriguingly however, Fe-S cluster protein function in skeletal muscle is strikingly more affected by iron deficiency that in other tissues [10, 13]. As mentioned above, total IRP1 RNA binding activity is decreased in skeletal muscle in response to iron deficiency, but unaffected in the liver (Clarke, unpublished data). Similarly, c-acon activity is unchanged in the liver, but significantly decreased in the muscles of iron deficient rats [10, 104]. Previous studies have also demonstrated significant reductions in mitochondrial enzyme activity, Fe-S enzyme content, and IscS protein abundance in skeletal muscle in response to dietary iron deficiency [10-12]. Currently, the mechanisms underlying the tissue-specific responses to iron deficiency remain unknown, but the recent discovery of a new class of small regulatory molecules called microRNA (miRNA) has begun to receive a lot of attention

as miRNA because of their tissue-specific expression patterns and anticipated participation in nearly every biological process within the cell.

A brief introduction to microRNA

The first miRNA, lin-4, was discovered in 1993, and was identified as being critically important for developmental timing in *Caernorhabditis elegans*, although at the time it was largely considered an anomaly in worm genetics [105]. The next miRNA discovered, let-7, was not identified until 2000 [106]. Intriguingly, the miRNA let-7, also discovered in *C. elegans*, was found to be highly conserved among all animals [107]. Shortly thereafter, in 2001, several additional miRNA were discovered in *Drosophila melanogaster* and in the human HeLa cell line [108, 109]. To date (June 2013), 21,264 precursor miRNA expressing 25,141 mature miRNA have been annotated in 193 species and logged in the latest (release 19) miRBase database repository [110]. The distinction between precursor and mature miRNA is discussed below. With thousands of miRNA in numerous species being identified in a relatively short period of time, it was necessary to establish criteria to be used in annotating each newly discovered miRNA [111, 112]. Each experimentally validated novel miRNA is designated with a unique name following these rules prior to publication. Exceptions have been made for the miRNA let-7 and lin-4. whose names have been retained for historical reasons.

First, miRNA are labeled numerically, and in sequential order with the prefix "mir" followed by a dash, with an un-capitalized "mir-" generally referring to the precursor miRNA, while a capitalized "miR-" generally denotes the mature form. For instance, if the last annotated human precursor miRNA was mir-6724, the next novel published miRNA precursor will be numbered miR-6725. For further clarification, the

names are also preceded by 3 letters signifying the species of origin, such as “hsa-” for *Homo sapiens*, “mmu-” for *Mus musculus*, or “dme-” *Drosophila melanogaster*.

Additionally, miRNA with nearly identical structure and sequencing, barring one or two nucleotides, are annotated with a lower case letter such that relationships among miRNA can be inferred (e.g., miR-181a is closely related to miR-181b) [112]. Numbered suffixes, however, designate distinct precursor and genomic loci that express 100% identical mature miRNA [112]. For example, the designation of hsa-mir-6725-1 and hsa-mir-6725-2 would indicate that while these two precursor miRNA may be located in different regions of the genome, both are processed into identical mature miRNA, hsa-miR-6725. miRNA which originate from the same precursor are often referred to as a miRNA:miRNA* (or miRNA-star) duplex [113]. With this star/non-star nomenclature, the non-star strand of the duplex represents the predominant functional “guide” strand, and the star strand represents the less abundant and more rapidly turning over “passenger” strand. However, when available sequencing data is not sufficient to designated the predominant strand, a naming convention that identifies the miRNA strand location on the 5’- or 3’-arm of the precursor miRNA is used (e.g., hsa-miR-6725-5p and hsa-6725-3p) [113].

microRNA biogenesis and processing

The majority of miRNA are derived from exons or introns of non-coding RNA, but approximately one-third are located in the introns of mRNA encoding genes [114]. About half of mammalian miRNA loci are located in close proximity to other miRNA on the genome [114, 115]. These so-called “clustered” miRNA are likely transcribed from the same polycistronic transcription unit, and can work in tandem to regulate a cohort of

related mRNA targets [115]. Mammalian miRNA are transcribed by RNA polymerase II as long primary miRNA (pri-miRNA) transcripts that contain at least one hairpin structure consisting of a double-stranded stem and a terminal loop, and may be several kilobases in length [116]. In the nucleus, the pri-miRNA is cleaved at the stem of the hairpin structure by the microprocessor core complex composed of the RNase II-type protein Drosha and its cofactor known as DiGeorge syndrome critical region gene 8 (DGCR8) [116, 117]. The product of this processing is an ~70 nucleotide (nt) long precursor miRNA (pre-miRNA) that is then exported out of the nucleus into the cytoplasm by the nuclear export factor exportin 5 (Exp5) through recognition of a short 3'-overhang [117]. Upon entry into the cytoplasm, the RNase III-like enzyme Dicer catalyzes the second processing step of "dicing" the pre-miRNA to produce an ~22 nt long miRNA duplex [117, 118].

Following cleavage by Dicer, the miRNA duplex is loaded onto an Argonaute (Ago) protein, which is a highly specialized small-RNA-binding protein and a critical component of RNA-silencing pathways [119]. Following loading onto an Ago protein, one of the two strands (generally the guide strand) is assembled into the RNA-induced silencing complex (RISC) to facilitate RNA silencing [120]. The loaded RISC is then competent to interact with recognition sites known as seed sequences typically located in the 3'UTR of target mRNA, though examples exist where the seed sequence is located in the 5'UTR or even within the open-reading frame of target mRNA [121, 122]. Upon binding to a target sequence, the RISC functions to silence the target mRNA via mRNA degradation or translational repression [116, 118]. The unloaded strand, often referred to as the passenger or miRNA* strand, was initially thought to be removed from the RISC

and degraded, but recent work indicates that these so-called miRNA* strands also have important functional regulatory roles [123, 124]. **Figure 1** represents an overview of basic mammalian miRNA biogenesis and function.

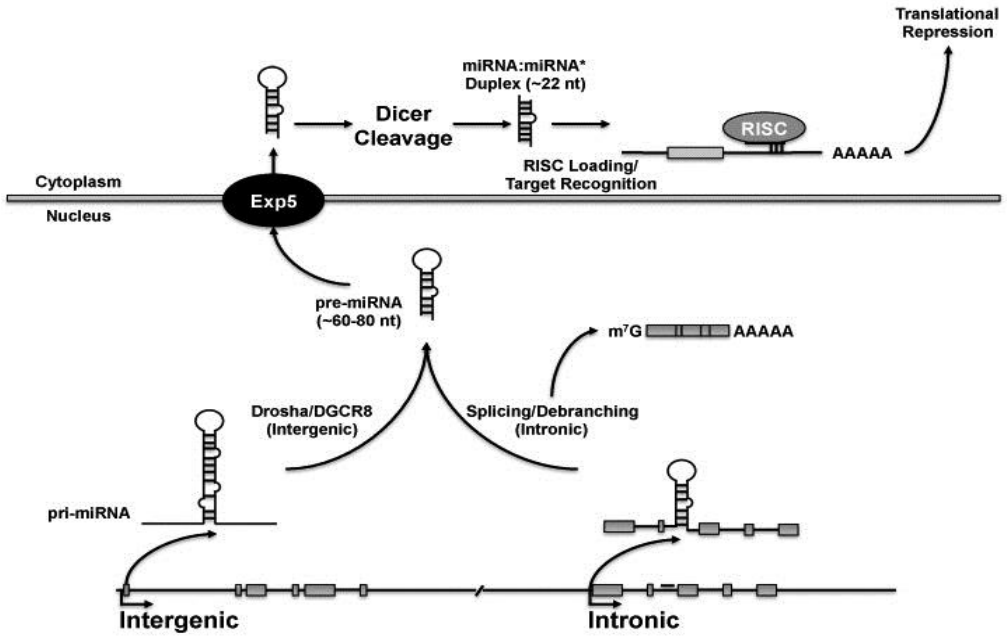

Figure 1. Overview of mammalian miRNA biogenesis and function. The primary (pri-) miRNA transcripts that can adopt hairpin-like structures are transcribed from miRNA loci. Pri-miRNA transcripts from miRNA genes are processed to 60-80 nt pre-miRNA transcripts by a complex containing Drosha and DGCR8 in the nucleus. Alternatively, pre-miRNA may be derived from intronic regions of protein-coding genes in a Drosha/DGCR8 independent process requiring both the splicesome and a debranching enzyme known as the lariat debranching enzyme. Both the canonical Drosha-dependent processing and intronic processing pathways generate a pre-miRNA with a hairpin-like structure that is then exported out of the nucleus into the cytoplasm through Exportin 5 (Exp5). Along with Argonaute (Ago) proteins, Dicer processes the pre-miRNA transcript into a mature miRNA duplex. The strand in the duplex with the least thermodynamically stable 5' end (guide strand) is retained by an Ago protein in mammals. The passenger strand (miRNA*) is generally released and degraded. Upon target recognition by the RNA-induced silencing complex (RISC) based on the seed region complementarity with the target mRNA, the target mRNA undergoes translational repression. From: Clarke, Stephen L., McKale R. Davis, and Ramanjulu Sunkar. "Biogenesis of Mammalian miRNA." *MicroRNAs as Tools in Biopharmaceutical Production*. Springer Netherlands, 2012. 15-27.

Interestingly, iron appears to play a critical role in miRNA processing via its physiological role as the functional component in heme. This potential role for iron to participate in miRNA biogenesis was first demonstrated when DGCR8 was identified as a heme-binding protein [26]. Additional studies demonstrated that heme-free DGCR8 was less active than heme-bound DGCR8 and suggests that an impaired ability to synthesize heme as a result of inadequate iron could decrease pri-miRNA processing [26]. In addition to heme availability, the oxidation state of iron in heme affects heme-mediated regulation of DGCR8 [25]. The reduction of ferric heme to the ferrous heme abolishes DGCR8 pri-miRNA processing activity thereby affecting the rate and efficiency of pri-miRNA processing [25]. Recent work has now provided evidence that iron also regulates the processing of pre-miRNA via the iron-dependent regulation of Dicer activity through its association with poly(C)-binding protein 2 (Pcbp2) [125]. Pcbp2 association with Dicer appears to promote cytosolic processing of pre-miRNA precursors [125]. The effect of Pcbp2 on pre-miRNA processing was enhanced with the removal of cytosolic iron, but not heme-iron, via the use of iron chelators [125]. **Figure 2** illustrates key aspects of miRNA processing that may be influenced by iron availability.

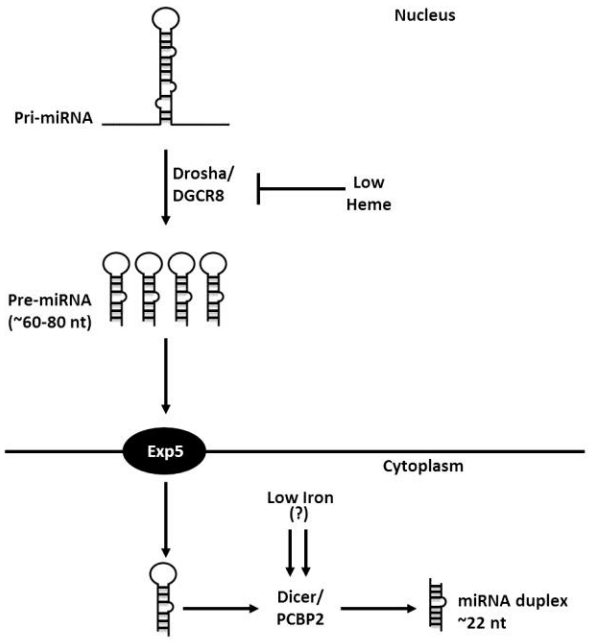

Figure 2. Relationship between cellular iron and miRNA processing. Following their transcription, primary miRNA (pri-miRNA) are cleaved at the stem of the hairpin structure by the RNase II-type protein Drosha and its cofactor DGCR8, a heme-binding protein. Heme-free DGCR8 is less active than heme-bound DGCR8 suggesting that cellular iron status may affect the rate and efficiency of pri-miRNA processing. The product of Drosha/DGCR8 processing is a ~70 nt long precursor miRNA (pre-miRNA) that is exported out of the nucleus into the cytoplasm by the nuclear export factor exportin 5 (Exp5) through the recognition of a short 3'-overhang on the pre-miRNA. Upon entry into the cytoplasm, the RNase III-like enzyme Dicer catalyzes the second processing step of "dicing" the pre-miRNA to produce a ~22 nt long miRNA duplex. Preliminary evidence suggests that iron also regulates the processing of pre-miRNA via the iron-dependent regulation of Dicer activity through its association with poly(C)-binding protein 2 (PCBP2), wherein the removal of cytosolic iron, but not heme-iron, enhances pre-miRNA processing. Following cleavage by Dicer, the miRNA duplex is available to be assembled into the RISC to participate in RNA silencing of target mRNA.

microRNA function

miRNA are now recognized as the largest subclass of non-coding RNA and are predicted to regulate anywhere from 30% to as much as 60% of all protein-coding genes [27]. Indeed, the importance of miRNA has been confirmed in various cellular processes including cell fate determination, development, proliferation, and apoptosis, and miRNA are now thought to participate in nearly every biological aspect within the cell [116]. Underscoring their importance in the maintenance of proper cell function is the fact that misregulation of miRNA has been implicated in the pathogenesis of many human diseases such as cancer and metabolic disorders [126, 127]. miRNA regulate gene expression by promoting mRNA degradation, inhibiting translation, or both [118]. Under normal physiologic conditions, miRNA act as rheostat-like regulators that serve to fine tune gene expression, whereas as under pathologic conditions such as stress or disease they tend to display a much more pronounced function.

Since their discovery in 1993, remarkable progress has been made in our understanding of miRNA biogenesis, processing, and function. However, the details surrounding the mechanisms by which miRNA confer their function remain somewhat unclear. In order to understand the function of a miRNA, it is first necessary to identify the genes that it regulates. Unfortunately, identification of miRNA targets is a rather arduous task for several reasons. Foremost, the rules of targeting are not completely understood [128]. Also, target identification in mammals is quite challenging because miRNA bind to their target mRNA with only partial complementarity over a very short sequence, and suppression of a target gene is often quite small [129-131]. Furthermore, an individual miRNA can potentially regulate hundreds of genes, and ~60% of mRNA

have predicted binding sites for one or multiple miRNA. Thus, identification of miRNA gene targets is one of the most tedious aspects of miRNA research.

Several miRNA target features are important in determining miRNA:mRNA interaction and miRNA function. The most important feature for miRNA target recognition is Watson-Crick pairing of nucleotides 2-8 from the 5' end of the miRNA, known as the "seed" sequence with corresponding sites in target mRNA, referred to as "seed sites" [129, 131]. A "stringent" seed site has perfect Watson-Crick pairing of all 8 nucleotides. Bulges and mismatches, or "moderate" seed matching are also functional because RISC can tolerate small mismatches, or G:U wobble pairing, within the seed region [129]. Relative hierarchical efficacies of these seed matches are as follows: stringent seed > moderate stringent seed > bulge > G:U wobble [129]. Multiple seed sites within the target mRNA are also more efficient than single sites, and tend to exhibit additive effects [130]. Other features to consider when attempting to identify miRNA targets are site location and site accessibility. Although functional and efficacious miRNA sites have been identified within the coding sequence (CDS) and 5'UTR regions, miRNA sites tend to be preferentially located in the 3'UTR [121, 122, 129]. This is likely because RISC competes with other protein complexes, such as ribosomes and translation initiation complexes located in the CDS and 5'UTR, respectively, making the 3'UTR more accessible for binding [129]. Secondary structure of mRNA can also interfere with miRNA:mRNA interaction, and so minimum free energy can also be used to estimate site accessibility, but from a practical standpoint, the amount of A:Us surrounding the site can be an equally useful approach [129].

Once a miRNA target site has been identified, functional assays examining the effects of the miRNA on target gene expression is the next step in validating a *bona fide* miRNA target. The most common approach is to clone the region of the mRNA believed to be targeted into a luciferase reporter [131]. This reporter construct can then be co-transfected with a mimic or inhibitor of the miRNA of interest, and functionality can be assessed by either a decrease or increase in luciferase activity, respectively. Examination of miRNA regulation of a target mRNA *in vivo* is somewhat more difficult because under physiologic conditions miRNA only elicit modest reductions in target gene expression [131]. Furthermore, it was initially thought that miRNA only repressed target translation in mammals, making high-throughput approaches for assessing miRNA targets quite difficult [118]. However, it is now recognized that miRNA can induce mRNA degradation, and that this is likely the primary means of target gene regulation in mammals [118, 132]. These findings are quite exciting because of the enhanced sensitivity and high-throughput capabilities of whole-transcriptome analyses techniques.

Even now, while we are still making vast attempts to enhance our understanding of how miRNA target regulation works, recent developments have added a new twist in the miRNA:mRNA relationship. It is now recognized that targets can actually reciprocally regulate miRNA stability and miRNA function [128]. Curiously, target-sequence interactions can both enhance miRNA stability and stimulate miRNA degradation. For instance, in *C. elegans,* the AGO homolog ALG-1 protects miRNA from the 5'-to-3' exonucleases XRN-1 and XRN-2 thereby stabilizing miRNA abundance [128]. However, in *D. melanogaster* and human cells, extensive pairing between a miRNA and its target site induces 3' end trimming of the miRNA thereby enhancing

miRNA decay [128]. Furthermore, miRNA function can also be repressed by target mRNA without actually inducing changes in miRNA levels. Large-scale analysis of protein-coding mRNA and miRNA expression profiles has provided evidence that thousands of transcripts may actually be acting as target decoys [128]. These so called competing endogenous RNA (ceRNA) function by presenting target sites for miRNA binding, and titrating them from other target mRNA. Long non-coding RNA, pseudogene RNA, and mRNA can all act as ceRNA [128]. For example, zinc finger E-box-binding homeobox 2 (ZEB2), contains common miRNA binding sites with the tumor suppressor gene PTEN, and increased expression of ZEB2 mRNA was shown to sequester repressive miRNA from PTEN, subsequently preventing the miRNA-induced repression of PTEN gene expression [133].

Although our understanding of miRNA function and regulation has increased tremendously since the discovery of the first miRNA nearly 20 years ago, many questions still remain surrounding the cellular conditions and environmental cues that trigger specific miRNA responses. Provided the increasing roles for miRNA in fine-tuning gene expression and coordinating cellular functions, it is reasonable to speculate that nutrient availability or nutritional status might also affect miRNA expression in an effort to maintain nutrient homeostasis. Thus characterizing factors that contribute to alterations in miRNA biogenesis and processing will enhance our understanding of mechanisms by which cells respond to alterations to various situations such as changes in environmental conditions and nutrient availability.

microRNA and iron homeostasis

To date, an investigation into the extent to which dietary iron influences miRNA expression or regulation has not been fully described. Despite the paucity of data in terms of altered miRNA expression in response to dietary intake, there is ample evidence indicating a potential role for miRNA to regulate both systemic and cellular iron homeostasis at multiple points by influencing iron absorption, transport, storage, and utilization (see **Figure 3**). For instance, iron absorption and utilization may be affected by repression of the non-IRE isoform of DMT1 by miR-let-7d [134]. Overexpression of miR-let-7d in K562 erythroleukemia cells suppress expression of both DMT1 (non-IRE) mRNA and protein levels thereby decreasing the export of endosomal iron for use by the cell [134]. The decrease in endosomal iron export elicited an iron-deficient response, as evidenced by an increase in TfR expression, decreased ferritin protein abundance, and decreased hemoglobin content of the cell [134].

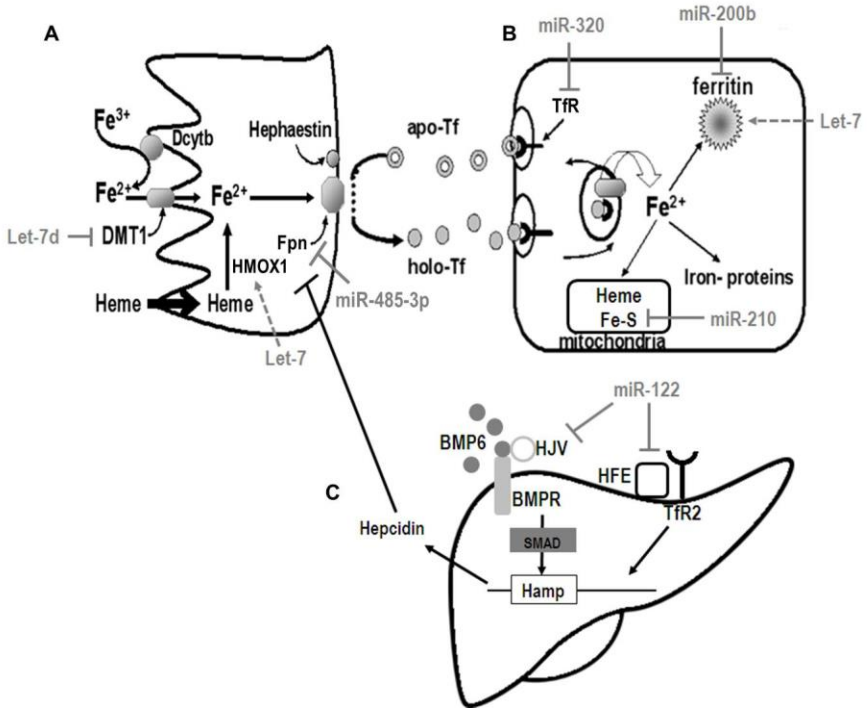

Figure 3. The potential roles for miRNA to influence iron metabolism. (**A**) Dietary iron absorption. Non-heme (Fe^{3+}) iron may be reduced by duodenal cytochrome B (Dcytb) and transported into the cytosol by divalent metal transporter-1 (Dmt1). Dietary heme iron is transported across the apical membrane by mechanisms that remain unclear and iron is released from heme by heme oxygenase (Hmox1). Hmox1 expression is de-repressed via let-7 targeting the transcriptional repressor Bach1. Iron that is neither stored nor utilized by the enterocyte is exported across the basolateral membrane by ferroportin-1 (Fpn) where it is oxidized by hephaestin before being bound to transferrin for transport to other tissues. Iron export can be repressed through direct inhibition of Fpn by miR-485-3p. (**B**) Cellular iron uptake. The transferrin-bound iron binds to the transferrin receptor (TfR) on the surface of the cell. The Tf/TfR complex is internalized through receptor-mediated endocytosis and upon acidification of the endosome results in iron release from Tf. The Tf/TfR complex can then be recycled back to the plasma membrane where the complex is dissociated at a neutral pH. MiR-320 contributes to the regulation of cellular iron uptake by repressing TfR translation to decrease transferrin-dependent iron uptake. Endosomal iron released from Tf is carried into the cytoplasm by Dmt1, the expression of which is repressed by the miRNA let-7d. The iron is then either stored in ferritin or utilized for iron-containing proteins. The regulatory action of let-7 on Bach1 to de-repress ferritin transcription potentially enhances cytosolic iron storage. Utilization of iron is influenced directly by miR-210 which targets the Fe-S cluster assembly proteins Iscu1/2 thereby decreasing mitochondrial metabolism. (**C**) Systemic control of iron homeostasis. In the liver, Tf interacts with TfR2 and the protein Hfe to trigger the bone morphogenetic protein (BMP) and SMAD signaling cascade via interaction with the BMP co-receptor hemojuvelin (Hjv) to activate Hamp (hepcidin) transcription. The liver specific miR-122 directly targets HFE and HJV to contribute to the regulation of systemic iron homeostasis by decreasing hepcidin mRNA expression.

Iron acquisition is also likely regulated by to miRNA-dependent mechanisms. For example, overexpression of miR-210 decreases TfR protein abundance in MCF7 cells [32]. Furthermore, the enhanced expression of miR-320 decreased the abundance of TfR on the plasma membrane and subsequent iron uptake in the lung carcinoma cell line A549 [135]. The multi-functional iron-binding protein lactoferrin, along with its receptor, is also regulated by miRNA in human cancer cells. Lactoferrin was characterized as a functional target of miR-214 in both HC11 and MCF7 cells [136]. Interestingly the seed region aligning to miR-214 in the 3'UTR of lactoferrin is very highly conserved and identical in the lactoferrin 3'UTR of mouse, rat, pig, goat, camel, bovine, and human species [136]. The post-transcriptional expression of the lactoferrin receptor is mediated by miR-584 in both Caco-2 cells and in mouse small intestine during the perinatal period [137]. Cellular export may also represent a miRNA-mediated regulation of iron homeostasis as the only known cellular iron exporter, Fpn, was recently shown to be targeted by miR-485-3p [138]. Overexpression of miR-485-3p resulted in increased cellular iron levels, while inhibition of miR-485-3p expression decreased cellular iron levels. In the absence of a regulated excretory pathway to rid the body of excess iron, the regulation of iron uptake or acquisition is a key point of control in maintaining cellular and systemic iron homeostasis. These exciting findings highlight the potential for miRNA to provide an additional means of control to fine-tuning the regulation of cellular iron uptake and export.

In addition to the regulation of iron uptake and acquisition, miRNA may also contribute to the control of cellular iron homeostasis through regulation of iron storage via ferritin. The expression of both forms of the iron storage protein ferritin, FtH and

FtL, are significantly higher in human breast cancer cells with a particularly aggressive phenotype and correlates with a decreased expression of miR-200b [139]. The de-repression of FtH expression may be, at least in part, due to the presence of a miR-200b seed sequence in FtH [139]. Interestingly, miR-200b has also been shown to correlate with dietary zinc depletion and repletion [140]. The functional and physiologic causes and consequences of miR-200b regulation in response to alterations in iron and zinc status will likely be the focus of future studies. Iron storage may also be indirectly affected by miRNA as both miR-196 and let-7d target the heme-regulated transcriptional repressor Bach 1, which results in a de-repression of Bach1 targets such as HMOX1 and ferritin [41, 141]. Although ferritin transcription may be reduced via Bach1, the capacity for let-7d-dependent repression of Bach1 to de-repress ferritin expression and synthesis remains unknown [142].

Systemic iron homeostasis is also likely influenced by miRNA expression via the liver-specific miR-122 [33]. Inhibition of miR-122 by locked nucleic acid (LNA) modification is associated with an increased expression of HFE, HJV, BMPR1A, and hepcidin mRNA, all of which contribute to a reduction in both plasma and liver iron, in addition to mildly impaired hematopoiesis [33]. In fact, both HFE and HJV are directly targeted by miR-122, suggesting that miR-122 could be targeted for therapeutic intervention for diseases of iron metabolism [33]. Intriguingly, miR-122 also correlates with copper accumulation and the onset of fulminant hepatitis in a rodent model of Wilson's disease [143]. Elevated serum levels of miR-122 are detectable as much as two weeks earlier than traditional hepatitis-associated serum markers and therefore may represent a potential non-invasive biomarker for early detection of liver disease [143].

While it is tempting to postulate that miR-122 may be yet another interesting link between iron and copper metabolism, it is important to note that miR-122 compromises ~70% of all hepatic miRNA expression, and is therefore likely to have numerous hepatic regulatory capacities [29, 34].

Erythropoietic demand for iron to support the synthesis of hemoglobin is another major factor in coordinating iron absorption and utilization, thus miRNA-dependent control of erythropoiesis has the potential to contribute to the control of systemic iron homeostasis. Interestingly, many miRNA are highly expressed in the initial stages of erythropoiesis and a decline in their expression is required for normal erythrocyte proliferation (miR-223), differentiation (miR-150), and maturation (miR-221/222) [144]. Conversely, miR-96 is actually more abundant in adult reticulocytes than umbilical cord blood, and contributes to the regulation of adult erythropoiesis via its direct interaction and repression of γ-globin [145]. The therapeutic potential for the manipulation of erythropoiesis via targeting of miRNA is the focus of considerable investigation.

Though the miRNA-dependent regulation of Fe-S cluster biogenesis and the potential effects on cellular iron metabolism via regulation of IRP1 has been suggested, the effects of dietary iron intake or iron status on miRNA expression and Fe-S cluster assembly have not been extensively investigated. Current evidence suggests that the hypoxia-inducible miR-210 targets Fe-S biogenesis and assembly via the regulation of the iron-sulfur cluster scaffold proteins Iscu1/2 [31, 32]. Given the overlap between iron and oxygen sensing and maintenance of iron homeostasis, the potential for miR210 to repress Fe-S cluster biogenesis, and thereby contribute to the regulation of IRP1 activity remains of considerable interest. To date however, the effect of miR-210 dependent

repression of Iscu1/2 expression on IRP1 function and its potential impact on cellular iron homeostasis is not yet fully characterized.

Despite the inherent challenges associated with interrogating the impact of nutrient status on miRNA expression and regulation, the pursuit of identifying these relationships between nutrient status (e.g., iron deficiency) and miRNA expression is warranted as the molecular mechanisms coordinating miRNA regulation and iron homeostasis are not yet fully understood or characterized. Finally, it remains to be established whether many of the miRNA demonstrated to affect iron metabolism using cell-based or other genetic approaches, such as miR-320 and miR-200b, have physiological roles *in vivo* or in non-transformed cell types, especially in response to physiologically-relevant alterations in nutrient intake.

CHAPTER III

ENHANCED EXPRESSION OF LIPOGENIC GENES MAY CONTRIBUTE TO HYPERGLYCEMIA AND ALTERATIONS IN PLASMA LIPIDS IN RESPONSE TO DIETARY IRON DEFICIENCY

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RESEARCH PAPER

Enhanced expression of lipogenic genes may contribute to hyperglycemia and alterations in plasma lipids in response to dietary iron deficiency

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Abstract Iron deficiency (ID) remains a public health concern affecting ~25% of the world's population. Metabolic consequences of ID include elevated plasma glucose concentrations consistent with increased reliance on glucose as a metabolic substrate, though the mechanisms controlling these responses remain unclear. To further characterize the metabolic response to ID, weanling male Sprague–Dawley rats were fed either a control (C; 40 mg Fe/kg diet) or iron-deficient (ID; 3 mg Fe/kg diet) diet or were pair-fed (PF) the C diet to the level of intake of the ID group for 21 days. In addition to reductions in hemoglobin, hematocrit, and plasma iron, the ID group also exhibited higher percent body fat and plasma triglycerides compared to the PF group. Steady-state levels of both plasma glucose and insulin increased 40 and 45%, respectively, in the ID group compared to the PF group. Plasma cortisol levels were decreased 67% in the ID group compared to the PF diet group. The systematic evaluation of the expression of genes involved in insulin signaling, glucose metabolism, and fatty acid metabolism in the liver and skeletal muscle revealed significant alterations in the expression of 48 and 52 genes in these tissues, respectively. A significant concurrent increase in lipogenic gene expression and decrease in gene expression related to β-oxidation in both the liver and skeletal muscle, in combination with differential tissue expression of genes involved in glucose metabolism, provides novel insight into the adaptive metabolic response in rodent models of severe iron deficiency anemia.

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Introduction

Iron is an essential nutrient as it is vital for many life-preserving processes including oxygen transport and DNA synthesis, as well as cellular proliferation and energy metabolism. Iron deficiency (ID) remains a major public health concern affecting some 1.6 billion people worldwide and significantly contributing to the global burden of disease (Worldwide prevalence of anaemia 1993–2005: WHO global database on anaemia 2008). Symptoms of ID include weakness, fatigue, listlessness, and a compromised immune response. In laboratory animal models of ID, another observed but less understood physiological response to an inadequate iron status is an elevation in plasma glucose levels (Borel et al. 1991; Davies et al. 1984). ID hyperglycemic animals also exhibit elevated insulin levels, increased insulin sensitivity, and altered glucose utilization in peripheral tissues, particularly skeletal muscle (Borel et al. 1993; Brooks et al. 1987; Farrell et al. 1988).

In addition to altered glucose utilization, significant changes in lipid homeostasis have been reported, though mechanistic studies investigating the utilization, uptake, and storage of lipids in ID animals have offered mixed results (Amine et al. 1976; Jain et al. 1982; Sherman et al. 1978). For instance, although hypertriglyceridemia is commonly reported in ID animals, tracer studies examining the extent to which these changes in plasma lipids are a result of iron-dependent effects on endogenous triacylglycerol synthesis remain unclear (Amine et al. 1976; Amine and Hegsted 1971; Jain et al. 1982; Sherman et al. 1978). Despite variable changes in hepatic triacylglycerol content in response to ID,

an increased abundance of lipid droplets has been observed in the skeletal muscles of ID rats (Ross and Eisenstein 2002; Sherman et al. 1978; Stangl and Kirchgessner 1998). The disparity of these reports may be due to strain differences and/or the degrees of diet-induced ID that appear to have significant effects on the metabolic response to an impaired iron status (Borel et al. 1991; Stangl and Kirchgessner 1998; Yamagishi et al. 2000). Therefore, even though lipemia and hyperglycemia have been described in ID animals, relatively few studies have focused on characterizing alterations in gene expression in the liver and skeletal muscle that might contribute to the metabolic responses observed in ID (Amine et al. 1976; Borel et al. 1991; Farrell et al. 1988; Linderman et al. 1994; Sherman et al. 1978; Collins 2006; Kamei et al. 2010; Tosco et al. 2010).

Recently, investigations into the coordination of the metabolic adaptation to ID in *Saccharomyces cerevisiae* have produced intriguing results demonstrating that targeted mRNA degradation via the coordination of two mRNA binding proteins drives the metabolic adaption to iron deprivation (Puig et al. 2005, 2008). Although the types of studies conducted in *S. cerevisiae* will likely prove more difficult using animal models, they do suggest that at least part of the metabolic adaption to ID occurs at the level of mRNA expression and stability. Therefore, in an effort to begin to further characterize the metabolic response to ID, we examined the expression of genes involved in glucose and fatty acid metabolism in both the liver and skeletal muscle. We hypothesized that dietary iron restriction would significantly alter metabolic gene expression and would correspond to metabolic adaptations observed in response to ID such as hyperglycemia and hypertriglyceridemia. Our results suggest that lipogenic gene expression is significantly up-regulated and that gene expression related to β-oxidation is significantly down-regulated in response to ID. The findings presented herein provide insight into the potential mechanisms contributing to the alterations in fuel utilization and energy metabolism associated with ID.

Study design and methods

Twenty-four 21-day-old weanling male Sprague–Dawley (Harlan, IN, USA) rats were housed individually in stainless-steel, wire-bottomed cages at the Oklahoma State University Laboratory Animal Research facility in a temperature- and humidity-controlled environment and maintained on a 12-h light:dark cycle with ad libitum access to deionized water. Rats in each group were allowed access to the control diet for 3 days prior to starting dietary treatments. After the acclimation period, rats were randomly assigned to one of three diet groups ($n = 8$/group) for 21 days: control (C; 40 mg Fe/kg diet), iron-deficient (ID; <3 mg Fe/kg diet), or pair-fed (PF; fed the control diet at the level of intake of the ID group). The powdered diets were purchased from Harlan Teklad (Madison, WI, USA; C-TD.89300 and ID-TD.80396) (Report of the American Institute of Nutrition ad hoc Committee on Standards for Nutritional Studies 1977). Individual body weights and food intake were measured daily. After the 21-day experimental period, rats were anesthetized with a mixture of ketamine/xylazine (75 mg ketamine and 7.5 mg xylazine/kg body weight). Body composition was determined by dual-energy X-ray absorptiometry (DXA, Hologic QDR Series 4500). Rats were then killed by exsanguination between 8:00 and 10:00 a.m., and tissues were excised, snap frozen in liquid nitrogen, and stored at −80°C until further analysis.

Assessment of iron status

Whole blood was collected from the abdominal aorta into EDTA-coated tubes and assayed for hemoglobin and hematocrit concentrations using an electronic hematology analyzer (Max M Model; Coulter Corporation). To obtain plasma, whole blood was collected into EDTA-coated tubes, mixed on a rotator at 25°C for 20 min, centrifuged at $800\times g$ for 20 min at 4°C, and then stored at −80°C until further analysis. Plasma iron was determined using an ELAN 9000 ICP-Mass Spectrometer (PerkinElmer, IL, USA). Hemolyzed samples were excluded from the analysis.

Metabolic indices

Plasma glucose and triglycerides were measured by previously described enzymatic methods using ACE glucose and triglyceride reagents, respectively, (Alfa Wassermann, NJ, USA) on an ACE Clinical Analyzer (In Vitro Diagnostic Products for Human Use, Proposed Establishment of Glucose 1974; Bucolo and David 1973). Plasma insulin (Crystal Chem Inc., IL, USA) and cortisol (R&D Systems, MN, USA) were measured by ELISA according to the manufacturer's instructions.

Pathway-focused PCR array and qPCR

Changes in gene expression were analyzed by pathway-focused insulin signaling, glucose metabolism, and fatty acid metabolism PCR arrays for rat (SABiosciences, MD). Briefly, total RNA was isolated from a portion of the liver or gastrocnemius muscle using STAT-60 (Tel-test, Inc., TX). The concentration of RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, DE, USA), and integrity of the RNA was determined by

examining 18S and 28S rRNA by agarose gel electrophoresis. The RNA was then treated with DNase I (Roche, IN, USA) and reverse-transcribed using SuperScript II (Invitrogen, CA, USA) in a final volume of 120 µL. The cDNA was used as a template for qPCR according to the array instructions using SYBR green chemistry on an ABI 7900HT system (Applied Biosystems, CA, USA). Array data were analyzed using SABiosciences RT^2 Profiler PCR Data Analysis software at http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php and were considered significant at ≥1.5-fold change and $P < 0.05$. This level of significance was set based on previous work examining the validity and sensitivity of PCR arrays (Gaj et al. 2008; Jae-Eun Pie et al. 2010; Swali et al. 2011). Findings from these studies indicate that transcriptional responses to dietary intervention(s) tend to be modest yet biologically meaningful, even when fold changes are <2.0 and/or when P values >0.05 (Gaj et al. 2008; Jae-Eun Pie et al. 2010; Swali et al. 2011). Relative quantitation for each gene was determined by normalizing to 5 housekeeping genes (RPLP1, HPRT1, RPL13A, LDHA, and ACTB) comparing the ID and PF groups using the $2^{-\Delta\Delta Ct}$ method (User Bulletin no. 2, Applied Biosystems). For gene expression analysis by qPCR, cDNA was prepared as described and analyzed using the $2^{-\Delta\Delta Ct}$ method with Cyclophilin B (Cyclo) as the invariant control.

Statistical analysis

Comparisons among the three treatment groups were made using one-way ANOVA followed by least significant difference as the post hoc test using SPSS software version 17.0 (IBM-SPSS, IL, USA). Student's t tests were employed to determine significance between the ID and PF groups for PCR arrays and qPCR analyses. All tests were done at the 95% confidence interval and presented as means ± SEM.

Results

Hemoglobin, hematocrit, and plasma iron were significantly reduced by 50, 55, and 74%, respectively, in the ID group compared to rats in either the PF and C groups (Table 1) ($P < 0.05$). Others have previously described that rats fed an iron-restricted diet have decreased food intake compared to rats fed a control diet, likely as a result of a diminished appetite in response to iron deficiency (Amine et al. 1970; Beard et al. 1995; Chen et al. 1997). In the present study, the ID group consumed less food than the C group, and rats in the PF and ID groups weighed ~10% less than the rats in the C group (Table 2) ($P < 0.05$). Based on the differences in food intake and body weight, the PF group was selected as the more appropriate control to ensure that observed biological changes were the result of iron deficiency and not simply due to decreased nutrient intake. Interestingly, despite no differences in lean body mass or total body mass between the ID and PF groups, rats in the ID group exhibited higher percent body fat and liver weights compared to the PF group (Table 2) ($P < 0.05$).

Table 1 Hematologic indices of iron status

Diet	Hemoglobin (g/L)	Hematocrit (%)	Plasma iron (µmol/L)
Control	135.0 ± 3.0[a]	37.0 ± 0.9[a]	57.7 ± 5.7[a]
Pair-fed	133.0 ± 2.0[a]	38.1 ± 0.5[a]	49.9 ± 6.5[a]
Iron-deficient	66.0 ± 2.0[b]	16.8 ± 0.7[b]	12.7 ± 3.0[b]

Values are means ± SEM, $n = 8$/group

[ab] Means in the same column not sharing common superscripts are significantly different among treatment groups, $P < 0.05$

Steady-state levels of both plasma glucose and plasma insulin were increased 1.4- and 1.6-fold, respectively, in the ID group compared to the PF group (Table 3) ($P < 0.05$). To determine whether a stress-mediated increase in cortisol levels in response to iron deficiency was partially responsible for contributing to hyperglycemia, plasma cortisol levels were assessed (Campos et al. 1998; Weinberg et al. 1980). Plasma cortisol levels in the ID group were reduced by 67% in comparison to the PF group ($P < 0.05$) (Table 3). Plasma triacylglycerols were 40% higher in the ID group compared to the PF group ($P < 0.05$) (Table 3).

Gene expression analyses from the insulin signaling array showed a significant increase in the expression of insulin-1 (INS1) and insulin-2 (INS2) mRNA in the livers

Table 2 Anthropometric data

Diet	Body wt. (g)	Liver wt. (g/100 g body wt.)	Lean mass (g)	Body fat (%)
Control	196.3 ± 4.2[a]	5.02 ± 0.1[a]	182.7 ± 3.3[a]	8.4 ± 0.4[a]
Pair-fed	177.5 ± 2.2[b]	4.05 ± 0.1[b]	168.3 ± 7.1[b]	7.0 ± 0.4[b]
Iron-deficient	180.6 ± 6.9[b]	4.68 ± 0.1[c]	167.4 ± 1.7[b]	8.5 ± 0.6[a]

Values are means ± SEM, $n = 8$/group

[abc] Means in the same column not sharing common superscripts are significantly different among treatment groups, $P < 0.05$

Table 3 Plasma levels of glucose, insulin, cortisol and triglycerides

Diet	Glucose (mmol/L)	Insulin (pmol/L)	Cortisol (nmol/L)	Triglycerides (mmol/L)
Control	16.2 ± 0.8^{a}	100.8 ± 15.5^{ab}	193.9 ± 32.9^{ab}	1.1 ± 0.1^{a}
Pair-fed	12.1 ± 0.9^{b}	93.6 ± 11.7^{b}	261.4 ± 31.3^{b}	0.5 ± 0.0^{b}
Iron-deficient	17.5 ± 1.0^{a}	147.8 ± 13.7^{a}	113.9 ± 32.3^{a}	0.8 ± 0.1^{c}

Values are means ± SEM, $n = 8$/group

[abc] Means in the same column not sharing common superscripts are significantly different among treatment groups, $P < 0.05$

of the ID rats ($P < 0.05$) (Table 4). Compared to the PF group, the ID group also exhibited an increase in the hepatic expression of lipogenic insulin target genes such as sterol regulatory element binding transcription factor (SREBF1), fatty acid synthase (FASN), and acetyl-CoA carboxylase alpha (ACACA) ($P < 0.05$) (Table 4). Interestingly, SREBF1 expression was also significantly increased in the skeletal muscle of the ID animals ($P < 0.05$) (Table 6). Changes in SREBF1 and FASN mRNA expression were validated by qPCR. In response to ID, hepatic FASN mRNA expression was increased ninefold, and SREBF1 expression was increased fourfold in both liver and muscle ($P < 0.05$) (Figs. 1, 2).

Results from the pathway array focused on genes related to glucose metabolism revealed a number of genes whose expression was differentially altered between liver and skeletal muscle in response to dietary ID. For example, the expression of pentose phosphate pathway enzymes such as

Table 4 Genes that increased in livers of iron-deficient rats compared to pair-fed rats

Gene name	Gene symbol	Fold regulation	*P* value
Insulin signaling array			
Insulin1	Ins1	+11.8	0.026
Fatty acid synthase	Fasn	+9.2	0.052
Insulin2	Ins2	+8.4	0.006
Glucose-6-phosphatase, catalytic subunit	G6pc	+4.0	0.008
Sterol regulatory element binding transcription factor 1	Srebf1	+2.9	0.082
Low density lipoprotein receptor	Ldlr	+2.9	0.012
Nitric oxide synthase 2, inducible	Nos2	+2.1	0.037
Acetyl-Coenzyme A carboxylase alpha	Acaca	+2.1	0.034
Neuropeptide Y	Npy	+1.8	0.047
V-akt murine thymoma viral oncogene homolog 2	Akt2	+1.5	0.017
Glycogen Synthase kinase 3 beta	Gsk3b	+1.5	0.016
Bcl2-like 1	Bcl2l1	+1.5	<0.001
Glucose metabolism array			
Amylo-1,6-glucosidase, 4-alpha-glucanotransferase	Agl	+5.9	0.035
Phosphorylase, glycogen, liver	Pygl	+5.0	0.001
Pyruvate dehydrogenase kinase, isozyme 1	Pdk1	+4.8	0.053
ATP citrate lyase	Acly	+4.7	0.005
Phosphoglycerate kinase 1	Pgk1	+4.0	<0.001
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	+3.3	0.038
Pyruvate kinase, liver and RBC	Pklr	+3.1	0.005
Glucose-6-phosphate dehydrogenase	G6pd	+2.5	0.014
Transkeletose	Tkt	+2.4	<0.001
Ribokinase	Rbks	+2.2	0.050
Pyruvate dehydrogenase kinase, isozyme 4	Pdk4	+2.1	0.008
Pyruvate dehydrogenase (lipoamide) beta	Pdhb	+1.9	0.035
Phosphofructokinase, liver	Pfkl	+1.8	0.004
Dihydrolipoamide dehydrogenase	Dld	+1.8	0.039
Transaldolase 1	Taldo1	+1.6	0.027

Fold regulation indicates relative fold change in mRNA abundance in the ID group compared to the PF group

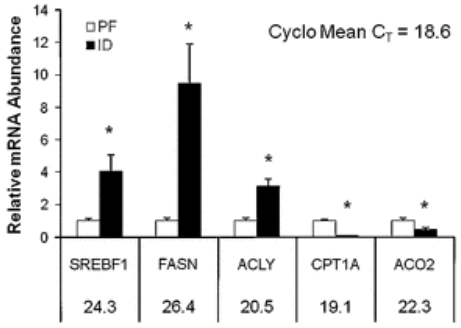

Fig. 1 Validation of select hepatic gene expression changes by qRT-PCR. mRNA levels were normalized to Cyclophilin B (Cyclo) mRNA as the invariant control. Numbers beneath gene names indicate C_T value obtained for the PF group. *Asterisk* indicates statistical significance between the PF and ID groups ($P < 0.05$). *Error bars* show SEM

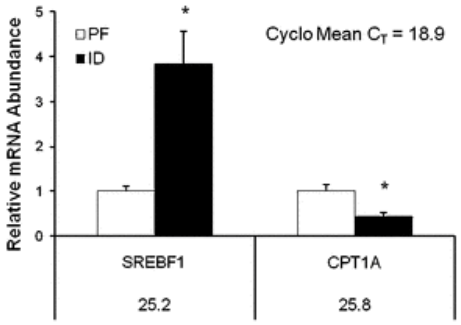

Fig. 2 Validation of select skeletal muscle gene expression changes by qRT-PCR. mRNA levels were normalized to Cyclophilin B (Cyclo) mRNA as the invariant control. Numbers beneath gene names indicate C_T value obtained for the PF group. *Asterisk* indicates statistical significance between the PF and ID groups ($P < 0.05$). *Error bars* show SEM

glucose-6-phosphate dehydrogenase (G6PD), transaldolase 1 (TALDO1), and transketolase (TKT) were increased in the livers of the ID group, while hexose-6-phosphate dehydrogenase (H6PD) and Taldo1 expression were decreased in the muscles of the ID group compared to the PF group ($P < 0.05$) (Tables 4, 7). Similarly, the expression of glycolytic genes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), and pyruvate kinase, (PKLR) were increased in the livers of ID rats, but the expression of the glycolytic genes Enolase-2 and -3 (ENO) were decreased in ID skeletal muscles ($P < 0.05$) (Tables 4, 7). Additionally, the expression of pyruvate dehydrogenase kinase 4 (PDK4) mRNA was significantly increased in the livers in the ID group, whereas it was significantly decreased in ID skeletal muscle compared to the PF group ($P < 0.05$) (Tables 4, 7).

Analysis of data from the fat metabolism array showed genes involved in β-oxidation, including acyl-CoA thioesterase 3 (ACOT3), carnitine palmitoyltransferase 1a (CPT1A), and carnitine palmitoyltransferase 1b (CPT1B), were significantly decreased in both the liver and skeletal muscle of the ID group ($P < 0.05$) (Tables 5, 7). The array findings for CPT1A were further examined by qPCR. CPT1A expression in the liver and skeletal muscles of the ID group was 90 and 54% lower, respectively, than that of the PF group ($P < 0.05$) (Figs. 1, 2). Concurrently, genes involved in the regulation of fatty acid synthesis such as acyl-CoA synthetase long-chain family member 5 (ACSL5) and acyl-CoA synthetase medium-chain family member 3 (ASCM3) were increased in the skeletal muscle in response to ID ($P < 0.05$) (Table 6). Compared to the PF group, the expression of the fatty acid transporter (FATP) genes solute carrier (SLC) family 27 member 1 (SLC27A1, FATP1) and SLC27A3 (FATP3) in the liver and SLC271A2 (FATP2) and SLC27A4 (FATP4) in the muscle were also significantly decreased in the ID group ($P < 0.05$) (Tables 5, 7).

Table 8 highlights key findings regarding changes in metabolic gene expression in response to dietary iron deficiency including significant decreases in β-oxidative gene expression, such as CPT1A, in both the liver and muscle. A concurrent increase in lipogenic gene expression was also observed in both of these tissues as hepatic FASN expression was increased, and SREBF1 expression was increased in both the liver and muscle of ID animals. Expression of the TCA cycle gene ACO2 was also significantly increased, while expression of the glycolytic gene PFKL was significantly decreased in both the liver and skeletal muscle of the ID group. Interestingly, expression of the insulin responsive gene PDK4 was significantly increased in the liver, but decreased in the skeletal muscle in response to dietary ID.

Discussion

The precise mechanisms responsible for the relative hyperglycemia associated with ID anemia remain unclear (Brooks et al. 1987; Davies et al. 1984; Farrell et al. 1988; Yamagishi and Komabayashi 2003). Increased plasma glucose levels may be attributed to a decreased capacity for aerobic metabolism resulting from severely repressed hemoglobin levels characteristic of anemia, though the extent to which this metabolic phenotype is dependent upon iron alone is unclear. Even a modest reduction in hemoglobin induced by dietary copper deficiency impairs

Table 5 Genes that decreased in livers of iron-deficient rats compared to pair-fed rats

Gene name	Gene symbol	Fold regulation	*P* value
Glucose metabolism array			
Aconitase 2, mitochondrial	Aco2	−2.3	<0.001
Fatty Acid Metabolism Array			
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	Hmgcs2	−3.0	0.016
Enoyl-Coenzyme A, hydratase/3-hydroxyacyl-Coenzyme A dehydrogenase	Ehhadh	−2.6	0.003
Acyl-CoA-thioesterase 3	Acot3	−2.5	0.016
Rattus norvegicus acyl-Coenzyme A dehydrogenase family, member 10	Acad10	−2.1	0.004
2,4-dienoyl CoA reductase 2, peroxisomal	Decr2	−2.0	0.007
Protein kinase, AMP-activated, gamma 3 non-catalytic subunit	Prkag3	−1.8	<0.001
Acetyl-Coenzyme A acyltransferase 1	Acaa1	−1.8	0.019
Enoyl-Coenzyme A hydratase, short chain 1, mitochondrial	Echs1	−1.8	0.012
Acyl-CoA synthetase long-chain family member 4	Acsl4	−1.7	<0.001
Lipoprotein lipase	Lpl	−1.7	0.019
Acyl-Coenzyme A dehydrogenase family, member 9	Acad9	−1.7	0.017
Carnitine palmitoyltransferase 1a, liver	Cpt1a	−1.6	0.032
Solute carrier family 27 (fatty acid transporter), member 1	Slc27a1	−1.6	0.005
3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	Hmgcl	−1.5	0.002
Acyl-CoA-thioesterase 9	Acot9	−1.5	0.025
Solute carrier family 27 (fatty acid transporter), member 3	Slc27a3	−1.5	0.051
Carnitine palmitoyltransferase 2	Cpt2	−1.5	0.023
Glutaryl-Coenzyme A dehydrogenase	Gcdh	−1.5	0.049
Lipase, hormone sensitive	Lipe	−1.5	0.019

Fold regulation indicates relative fold change in mRNA abundance in the ID group compared to the PF group

Table 6 Genes that increased in gastrocnemius muscles of iron-deficient rats compared to pair-fed rats

Gene name	Gene symbol	Fold regulation	*P* value
Insulin signaling array			
Sterol regulatory element binding transcription factor 1	Srebf1	+2.7	0.055
Harvey rat sarcoma virus oncogene, subgroup R	Rras2	+1.5	0.033
Phosphofructokinase, liver	Pfkl	+1.7	0.036
Fatty acid metabolism array			
Fatty acid binding protein 5, epidermal	Fabp5	+5.5	<0.001
Acyl-CoA synthetase long-chain family member 3	Acsl3	+4.5	<0.001
Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Prkag2	+3.6	<0.001
Acetyl-Coenzyme A acetyltransferase 2	Acat2	+3.1	0.002
Acyl-CoA synthetase medium-chain family member 3	Acsm3	+1.9	0.009
Protein kinase, cAMP-dependent, catalytic, alpha	Prkaca	+1.9	<0.001
Acyl-CoA synthetase long-chain family member 5	Acsl5	+1.7	0.003
Protein kinase, AMP-activated, beta 1 non-catalytic subunit	Prkab1	+1.7	0.042

Fold regulation indicates relative fold change in mRNA abundance in the ID group compared to the PF group

glucose tolerance, though this response is largely dependent on the primary source of dietary carbohydrate (Fields et al. 1983, 1984; Hassel et al. 1983). Because the animals in the present study were not fasted prior to sample and tissue collection, an impaired glucose tolerance, as opposed to an increase in fasting glucose levels, cannot be ruled out, though evidence from previous studies suggests that ID primarily alters fasting glucose levels (Borel et al. 1991; Brooks et al. 1987; Farrell et al. 1988). In fact, blood glucose levels are inversely related to hemoglobin levels, with more severe anemia associated with a more pronounced elevation in fasting blood glucose (Borel et al. 1991).

Table 7 Genes that decreased in gastrocnemius muscles of iron-deficient rats compared to pair-fed rats

Gene name	Gene symbol	Fold regulation	*P* value
Insulin signaling array			
Fructose-1,6- biphosphatase 1	Fbp1	−2.1	0.013
Eukaryotic translation initiation factor 4E binding protein 1	Eif4ebp1	−1.9	0.006
Bcl2-like 1	Bcl2l1	−1.9	0.000
Insulin Receptor	Insr	−1.8	0.018
V-akt murine thymoma viral oncogene homolog 1	Akt1	−1.5	0.049
Glucose metabolism array			
Pyruvate dehydrogenase kinase, isozyme 4	Pdk4	−4.4	0.021
Hexokinase 3, white cell	Hk3	−3.1	0.012
Frucotse = 6,6-bisphosphatase 2	Fbp2	−2.9	0.010
Enolase 3, beta, muscle	Eno3	−2.3	0.008
Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	H6pd	−2.2	0.036
Glucose 6 phosphatase, catalytic, 3	G6pc3	−2.2	0.024
Enolase 2, gamma, neuronal	Eno2	−2.2	0.0499
Phosphoglycerate mutase 2 (muscle)	Pgam2	−2.1	0.019
Galactose mutarotase (aldose 1-epimerase)	Galm	−2.1	0.002
Phosphoenolpyruvate carboxykinase 2	Pck2	−2.1	0.012
Glycogen synthase 1, muscle	Gys1	−2.2	0.051
Succinate-CoA ligase, GDP-forming, beta subunit	Suclg2	−2.0	0.017
Oxoglutarate dehydrogenase-like	Ogdhl	−1.9	0.037
Transaldolase 1	Taldo1	−1.9	0.025
Glycogen synthase kinase 3 alpha	Gsk3a	−1.7	0.031
Glycogen synthase kinase 3 beta	Gsk3b	−1.7	0.029
Isocitrate dehydrogenase 2 (NADP +), mitochondrial	Idh2	−1.6	0.014
Aconitase 2, mitochondrial	Aco2	−1.6	0.012
Isocitrate dehydrogenase 3 (NAD +) beta	Idh3b	−1.5	0.046
Fatty acid metabolism array			
Carnitine palmitoyltransferase 1a, liver	Cpt1a	−8.1	0.000
Carnitine palmitoyltransferase 2	Cpt2	−4.4	0.000
3-hydroxybutyrate dehydrogenase, type 2	Bdh2	−2.9	0.000
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	Hmgcs2	−2.8	0.000
Solute carrier family 27 (fatty acid transporter), member 2	Slc27a2	−2.5	0.000
Acyl-CoA thioesterase 12	Acot12	−2.3	0.005
2,4-dienoyl CoA reductase 1, mitochondrial	Decr1	−2.2	0.000
Acyl-CoA thioesterase 2	Acot2	−2.2	0.003
Acyl-Coenzyme A dehydrogenase family, member 11	Acad11	−2.2	0.003
Acetyl-Coenzyme A acyltransferase 2	Acaa2	−1.9	0.002
Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	Hadha	−1.9	0.002
Carnitine acetyltransferase	Crat	−1.7	0.009
Glycerol-3-phosphate dehydrogenase 2, mitochondrial	Gpd2	−1.7	0.006
Acyl-Coenzyme A oxidase 3, pristanoyl	Acox3	−1.6	0.015
Acyl-Coenzyme A dehydrogenase family, member 10	Acad10	−1.5	0.008
Solute carrier family 27 (fatty acid transporter), member 4	Slc27a4	−1.5	0.002

Fold regulation indicates relative fold change in mRNA abundance in the ID group compared to the PF group

With only a moderately depressed hemoglobin concentration, there is a concomitant decrease in the oxidative capacity of peripheral tissues and an increased reliance on the anaerobic metabolism as evidenced by the increased blood lactate levels observed in ID animals (Brooks et al. 1987; Farrell et al. 1988; Linderman et al. 1994).

Table 8 Key tissue changes in gene expression in response to a dietary iron deficiency potentially contributing to a pro-lipogenic phenotype

Gene	Liver	Skeletal muscle	Function
SREBF1	↑	↑	Lipogenesis
FASN	↑	NC	β-oxidation
CPT1A	↓	↓	β-oxidation
CPT2	↓	↓	β-oxidation
ACAD10	↓	↓	β-oxidation
HMGCS2	↓	↓	Ketogenesis
ACO2	↓	↓	TCA Cycle
PFKL	↑	↑	Glycolysis
PDK4	↑	↓	Gluconeogenesis

Thus, elevated blood glucose levels provide a means of adapting to decreased oxidative capacity by ensuring that ample substrate is available for energy production (Brooks et al. 1987; Farrell et al. 1988; Linderman et al. 1994).

In addition to elevated plasma glucose, ID animals also exhibit a relative elevation in plasma insulin. Interestingly, extrapancreatic insulin gene expression has previously been reported in the livers of hyperglycemic mice and in iron-deficient rats (Chen et al. 2010; Kojima et al. 2004; Kamei et al. 2010). The increased hepatic expression of INS1 and INS2 mRNA may contribute to the increased steady-state level of insulin observed in the present study and those reported by others in similar experimental models (Beard 2001; Borel et al. 1993; Chen et al. 2010; Farrell et al. 1988; Kojima et al. 2004). The increased insulin could serve to facilitate the entry of glucose into insulin-dependent tissues (e.g., skeletal muscle). Indeed, insulin sensitivity is enhanced in ID, particularly when hemoglobin falls to 60 g/L or below, allowing for both increased glucose clearance and disposal rates (Borel et al. 1993; Farrell et al. 1988; Linderman et al. 1994). Moderate elevations in both glucose and insulin levels observed in ID presents an interesting question: Why do blood glucose levels remain elevated if circulating levels of plasma insulin are increased and peripheral insulin sensitivity is enhanced?

In addition to stimulating insulin-dependent glucose uptake, insulin also coordinates glucose homeostasis through the regulation of gluconeogenic gene expression via the insulin-induced phosphorylation of the transcription factor forkhead box protein O1 (FOXO1) (Nakae et al. 2002). Phosphorylation of FOXO1 prevents its nuclear translocation resulting in decreased expression of target genes such as PCK1, G6PASE, and PDK4 (Granner et al. 1983; Nakae et al. 2002; Gross et al. 2008). In the fed state when insulin levels are elevated, insulin also activates hepatic lipogenesis through the increased expression of genes such as ACACA and FASN via the transcription factor SREBF1 (Horton et al. 2002; Shimomura et al. 1999b). In contrast to the fed state, chronic hyperinsulinemia may alter normal insulin signaling. Obese diabetic (ob/ob) mice with chronic hyperinsulinemia exhibit "mixed insulin resistance", where a combination of hepatic insulin resistance (e.g., impaired repression of gluconeogenesis) and sensitivity (e.g., enhanced lipogenesis) exists (Kerouz et al. 1997; Li et al. 2010; Shimomura et al. 2000).

This model of mixed insulin resistance likely results from a bifurcation of the insulin signaling pathway at mammalian target of rapamycin complex 1 (mTORC1) (Li et al. 2010). In this model, mTORC1 continues to activate the SREBF1-dependent increase in lipogenesis, while FOXO1 target genes are inadequately repressed due to impaired insulin signaling that results in decreased phosphorylation of FOXO1 (Li et al. 2010). Although hepatic mTORC1 and FOXO1 phosphorylation were not assessed in this study, alterations in hepatic gene expression are consistent with impaired insulin signaling in response to severe ID. The increased hepatic expression of SREBF1, ACACA, and FASN mRNA observed in the current study suggests that normal insulin signaling through mTorc1 was retained. Despite increased lipogenic gene expression and increased plasma triacylglycerols, hepatic expression of the FOXO1 target gene PDK4 was increased and may indicate an impairment in insulin signaling (i.e., insulin resistance) in this part of the signaling pathway. Potential differences in insulin signaling in response to ID may provide an explanation for how blood glucose levels remain elevated even when both serum insulin levels and peripheral insulin sensitivity are increased (Beard 2001; Borel et al. 1993; Farrell et al. 1988). Iron-dependent factors or mechanisms contributing to altered insulin signaling remain to be characterized.

Ectopic lipid deposition in liver and skeletal muscle as a result of inappropriately elevated lipogenesis in ID may contribute to a condition of relative lipotoxicity and subsequent insulin resistance. The increased expression of SREBF1 mRNA is of particular interest in light of its role in stimulating lipogenic gene expression thereby promoting lipid accumulation in both the liver and skeletal muscle (Ducluzeau et al. 2001; Guillet-Deniau et al. 2002; Horton et al. 2003; Shimomura et al. 1999a). An imbalance of lipogenic gene expression coupled with a decrease in the expression of genes involved in β-oxidation of fatty acids in the liver, and the skeletal muscle may enhance lipid accumulation in these tissues.

If a relative lipotoxicity contributes to insulin resistance in response to ID, then one prediction of this model is that abnormal fat accumulation and increased plasma fatty acids and triacylglycerols might be observed. Indeed, in animal models of severe ID, lipids accumulate in both liver

and skeletal muscle (Johnson et al. 1990; Sherman et al. 1978). Elevated plasma triacyglycerols are also observed in a severely ID animals (Amine et al. 1976; Sherman et al. 1978; Yamagishi et al. 2000). Although neither hepatic nor skeletal muscle lipid accumulation was assessed in the present study, overall body composition was examined. Despite a similarity in body mass between the ID and PF groups, total body fat was higher in the ID group compared to the PF group. Because the diets between the ID and PF groups were isocaloric and differed only in iron content, increased lipogenesis and improper storage of excess fat may be a metabolic consequence of ID.

It is difficult to predict whether this proposed ID-induced mixed insulin resistance would result in further negative metabolic consequences similar to those observed in metabolic syndrome and type 2 diabetes as other more pressing problems begin to arise after an extended time on a severely iron-restricted diet. In fact, examining the metabolic response to ID is complicated by the observation that mild to moderate levels of ID are not associated with the pronounced alterations in glucose homeostasis observed in models where hemoglobin levels fall to ≤60 g/L (Borel et al. 1993, 1991; Linderman et al. 1994). The degree of anemia in most clinical cases of ID anemia is not as extreme as those typically induced in animal models (McLean et al. 2009). Nonetheless, ID remains the single most common micronutrient deficiency affecting not only under-nourished populations, but over-nourished populations as well (Lecube et al. 2006; Pinhas-Hamiel et al. 2003; Yanoff et al. 2007). As overweight and obesity already place individuals at risk for metabolic disease, future studies may be required to investigate the potential of ID to exacerbate conditions where normal glucose and fat metabolism are already disrupted. In fact, recent studies have found that ID may be more prevalent in overweight children and adolescents, as well as obese postmenopausal women, populations that are already at increased risk for developing metabolic disease (Lecube et al. 2006; Nead et al. 2004; Pinhas-Hamiel et al. 2003).

In the present study, we offer insight to the potential factors involved in the hyperglycemia and hyperinsulinemia observed in rodent models of ID anemia. As ID animals shift their reliance from fat to glucose as the preferred metabolic substrate for peripheral tissues, this increased reliance on glucose may contribute to elevated blood glucose levels that subsequently promote enhanced insulin secretion. In severe ID anemia, plasma glucose levels remain elevated despite both increased peripheral insulin sensitivity and glucose clearance rates. Iron-dependent alterations in glucose and lipid metabolism observed by us and others are consistent with a bifurcated insulin signaling pathway in the liver resulting in the continuous activation of lipogenesis, and thereby contributing to ectopic lipid accumulation and potential lipotoxicity. While the lipogenic pathway remains insulin-sensitive, the hepatic FOXO1 branch of the pathway becomes more insulin-resistant, resulting in a derepression of PDK4 gene expression subsequently increasing the availability of gluconeogenic substrate. Indeed, enhanced gluconeogenesis has been observed as a result of dietary iron deficiency (Borel et al. 1993; Linderman et al. 1994). Although much remains to be known about molecular mechanisms underlying the metabolic response to iron deficiency, the results presented herein provide evidence consistent with altered hepatic insulin signaling promoting enhanced lipogenesis and impaired lipid oxidation. Future studies further interrogating changes in the hepatic insulin signaling pathway in response to iron deficiency will enhance our understanding of metabolic adaptations that occur as a result inadequate iron intake.

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Conflict of interest None.

References

Amine EK, Hegsted DM (1971) Iron deficiency lipemia in the rat and chick. J Nutr 101(11):1575–1582

Amine EK, Corey J, Hegsted DM, Hayes KC (1970) Comparative hematology during deficiencies of iron and vitamin A in the rat. J Nutr 100(9):1033–1040

Amine E, Desilets EJ, Hegsted DM (1976) Effects of dietary fats on lipogenesis in iron deficiency anemic chicks and rats. J Nutr 106:405–411

Beard JL (2001) Iron biology in immune function, muscle metabolism and neuronal functioning. J Nutr 131(2S-2):568S–579S discussion 580S

Beard JL, Zhan CS, Brigham DE (1995) Growth in iron-deficient rats. Proc Soc Exp Biol Med Soc (New York, NY) 209(1):65–72

Borel MJ, Smith SH, Brigham DE, Beard JL (1991) The impact of varying degrees of iron nutriture on several functional consequences of iron deficiency in rats. J Nutr 121(5):729–736

Borel MJ, Beard JL, Farrell PA (1993) Hepatic glucose production and insulin sensitivity and responsiveness in iron-deficient anemic rats. Am J Physiol 264(3 Pt 1):E380–E390

Brooks GA, Henderson SA, Dallman PR (1987) Increased glucose dependence in resting, iron-deficient rats. Am J Physiol 253(4 Pt 1):E461–E466

Bucolo G, David H (1973) Quantitative determination of serum triglycerides by the use of enzymes. Clin Chem 19(5):476–482

Campos MS, Barrionuevo M, Alferez MJ, Gomez-Ayala AE, Rodriguez-Matas MC, Lopez Aliaga I, Lisbona F (1998) Interactions among iron, calcium, phosphorus and magnesium in the nutritionally iron-deficient rat. Exp Physiol 83(6):771–781

Chen OS, Schalinske KL, Eisenstein RS (1997) Dietary iron intake modulates the activity of iron regulatory proteins and the

abundance of ferritin and mitochondrial aconitase in rat liver. J Nutr 127(2):238–248

Chen X, Larson CS, West J, Zhang X, Kaufman DB (2010) In vivo detection of extrapancreatic insulin gene expression in diabetic mice by bioluminescence imaging. PloS one 5(2):e9397

Collins JF (2006) Gene chip analyses reveal differential genetic responses to iron deficiency in rat duodenum and jejunum. Biol Res 39(1):25–37

Davies KJ, Donovan CM, Refino CJ, Brooks GA, Packer L, Dallman PR (1984) Distinguishing effects of anemia and muscle iron deficiency on exercise bioenergetics in the rat. Am J Physiol 246(6 Pt 1):E535–E543

Ducluzeau PH, Perretti N, Laville M, Andreelli F, Vega N, Riou JP, Vidal H (2001) Regulation by insulin of gene expression in human skeletal muscle and adipose tissue. Evidence for specific defects in type 2 diabetes. Diabetes 50(5):1134–1142

Farrell PA, Beard JL, Druckenmiller M (1988) Increased insulin sensitivity in iron-deficient rats. J Nutr 118(9):1104–1109

Fields M, Ferretti RJ, Smith JC Jr, Reiser S (1983) Effect of copper deficiency on metabolism and mortality in rats fed sucrose or starch diets. J Nutr 113(7):1335–1345

Fields M, Ferretti RJ, Smith JC Jr, Reiser S (1984) Impairment of glucose tolerance in copper-deficient rats: dependency on the type of dietary carbohydrate. J Nutr 114(2):393–397

Gaj S, Eijssen L, Mensink RP, Evelo CT (2008) Validating nutrient-related gene expression changes from microarrays using RT(2) PCR-arrays. Genes Nutr 3(3–4):153–157

Granner D, Andreone T, Sasaki K, Beale E (1983) Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin. Nature 305(5934):549–551

Gross DN, van den Heuvel AP, Birnbaum MJ (2008) The role of FoxO in the regulation of metabolism. Oncogene 27(16):2320–2336

Guillet-Deniau I, Mieulet V, Le Lay S, Achouri Y, Carre D, Girard J, Foufelle F, Ferre P (2002) Sterol regulatory element binding protein-1c expression and action in rat muscles: insulin-like effects on the control of glycolytic and lipogenic enzymes and UCP3 gene expression. Diabetes 51(6):1722–1728

Hassel CA, Marchello JA, Lei KY (1983) Impaired glucose tolerance in copper-deficient rats. J Nutr 113(5):1081–1083

Horton JD, Goldstein JL, Brown MS (2002) SREBPs: transcriptional mediators of lipid homeostasis. Cold Spring Harb Symp Quant Biol 67:491–498

Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL (2003) Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc Natl Acad Sci USA 100(21):12027–12032

In Vitro Diagnostic Products for Human Use, Proposed Establishment of Glucose (1974). US Dept. of Health, Education and Welfare, FDA

Jae-Eun Pie Y-RK, Kim In-Kyoung, Seo Sang-Hui, Lee Seung Ho, Lee Hee Ra, Yoo Young, Chung Ji Tae, Youn Jong Pil, MoonJu Oh, Hwang Seung Yong, Kim Meyoung-Kon (2010) Correlation between nutrition intake and gene expression profiles in children with asthma. Mol Cell Toxicol 6:313–319

Jain SK, Yip R, Pramanik AK, Dallman PR, Shohet SB (1982) Reduced plasma cholesterol esterifying activity in iron-deficient rats: its possible role in the lipemia of iron deficiency. J Nutr 112(6):1230–1232

Johnson JA, Willis WT, Dallman PR, Brooks GA (1990) Muscle mitochondrial ultrastructure in exercise-trained iron-deficient rats. J Appl Physiol 68(1):113–118

Kamei A, Watanabe Y, Ishijima T, Uehara M, Arai S, Kato H, Nakai Y, Abe K (2010) Dietary iron-deficient anemia induces a variety of metabolic changes and even apoptosis in rat liver: a DNA microarray study. Physiol Genom 42(2):149–156

Kerouz NJ, Horsch D, Pons S, Kahn CR (1997) Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. J Clin Invest 100(12):3164–3172

Kojima H, Fujimiya M, Matsumura K, Nakahara T, Hara M, Chan L (2004) Extrapancreatic insulin-producing cells in multiple organs in diabetes. Proc Natl Acad Sci USA 101(8):2458–2463

Lecube A, Carrera A, Losada E, Hernandez C, Simo R, Mesa J (2006) Iron deficiency in obese postmenopausal women. Obesity (Silver Spring, MD) 14(10):1724–1730

Li S, Brown MS, Goldstein JL (2010) Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. Proc Natl Acad Sci USA 107(8):3441–3446

Linderman JK, Brooks GA, Rodriguez RE, Dallman PR (1994) Maintenance of euglycemia is impaired in gluconeogenesis-inhibited iron-deficient rats at rest and during exercise. J Nutr 124(11):2131–2138

McLean E, Cogswell M, Egli I, Wojdyla D, de Benoist B (2009) Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993–2005. Pub Health Nutr 12(4):444–454

Nakae J, Biggs WH 3rd, Kitamura T, Cavenee WK, Wright CV, Arden KC, Accili D (2002) Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nat Genet 32(2):245–253

Nead KG, Halterman JS, Kaczorowski JM, Auinger P, Weitzman M (2004) Overweight children and adolescents: a risk group for iron deficiency. Pediatrics 114(1):104–108

Pinhas-Hamiel O, Newfield RS, Koren I, Agmon A, Lilos P, Phillip M (2003) Greater prevalence of iron deficiency in overweight and obese children and adolescents. Int J Obes Relat Metab Disord 27(3):416–418

Puig S, Askeland E, Thiele DJ (2005) Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. Cell 120(1):99–110

Puig S, Vergara SV, Thiele DJ (2008) Cooperation of two mRNA-binding proteins drives metabolic adaptation to iron deficiency. Cell Metab 7(6):555–564

Report of the American Institute of Nutrition ad hoc Committee on Standards for Nutritional Studies (1977). J Nutr 107(7):1340–1348

Ross KL, Eisenstein RS (2002) Iron deficiency decreases mitochondrial aconitase abundance and citrate concentration without affecting tricarboxylic acid cycle capacity in rat liver. J Nutr 132(4):643–651

Sherman AR, Guthrie HA, Wolinsky I, Zulak IM (1978) Iron deficiency hyperlipidemia in 18-day-old rat pups: effects of milk lipids, lipoprotein lipase, and triglyceride synthesis. J Nutr 108(1):152–162

Shimomura I, Bashmakov Y, Horton JD (1999a) Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. J Biol Chem 274(42):30028–30032

Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, Goldstein JL (1999b) Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. Proc Natl Acad Sci USA 96(24):13656–13661

Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, Goldstein JL (2000) Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. Mol cell 6(1):77–86

Stangl GI, Kirchgessner M (1998) Different degrees of moderate iron deficiency modulate lipid metabolism of rats. Lipids 33(9):889–895

Swali A, McMullen S, Hayes H, Gambling L, McArdle HJ, Langley-Evans SC (2011) Cell cycle regulation and cytoskeletal remodelling are critical processes in the nutritional programming of embryonic development. PloS One 6 (8):e23189

Tosco A, Fontanella B, Danise R, Cicatiello L, Grober OM, Ravo M, Weisz A, Marzullo L (2010) Molecular bases of copper and iron deficiency-associated dyslipidemia: a microarray analysis of the rat intestinal transcriptome. Genes Nutr 5(1):1–8

Weinberg J, Dallman PR, Levine S (1980) Iron deficiency during early development in the rat: behavioral and physiological consequences. Pharmacol Biochem Behav 12(4):493–502

Worldwide prevalence of anaemia 1993–2005 : WHO global database on anaemia (2008). WHO

Yamagishi H, Komabayashi T (2003) Alteration of glucose metabolism and increased fructosamine in iron-deficiency anemic rats. Nutr Res 23(11):1547–1553

Yamagishi H, Okazaki H, Shimizu M, Izawa T, Komabayashi T (2000) Relationships among serum triacylglycerol, fat pad weight, and lipolysis in iron-deficient rats. J Nutr Biochem 11(9):455–460

Yanoff LB, Menzie CM, Denkinger B, Sebring NG, McHugh T, Remaley AT, Yanovski JA (2007) Inflammation and iron deficiency in the hypoferremia of obesity. Int J Obes (2005) 31(9):1412–1419

CHAPTER IV

COMPARISONS OF THE IRON DEFICIENT METABOLIC RESPONSE IN RATS FED EITHER AN AIN-76 OR AIN-93 BASED DIET

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Comparisons of the iron deficient metabolic response in rats fed either an AIN-76 or AIN-93 based diet

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Abstract

Background: Previous studies examining the metabolic consequences of dietary iron deficiency have reported elevated serum glucose concentrations in iron-deficient animals. Importantly, the majority of these findings were observed using an earlier version of a laboratory animal diet (AIN-76A) in which the primary carbohydrate source was sucrose – a disaccharide known to negatively impact both glucose and lipid homeostasis. The AIN-76A diet formula was improved in 1993 (AIN-93) to optimize animal nutrition with a major change being the substitution of cornstarch for sucrose. Therefore, we sought to examine the effects of iron deficiency on steady-state glucose homeostasis and the hepatic expression of glucose- and lipid-related genes in rats fed an iron-deficient diet based on either an AIN-76A or AIN-93 diet.

Methods: The study design consisted of 6 treatment groups: control (C; 40 mg Fe/kg diet), iron deficient (ID; ≤ 3 mg Fe/kg diet), or pair-fed (PF; 40 mg Fe/kg) fed either an AIN-76A or AIN-93 diet for 21 d. Hemoglobin and hematocrit were measured in whole blood. Serum insulin and cortisol were measure by ELISA. Serum glucose and triacylglycerols were measured by standard colorimetric enzyme assays. Alterations in hepatic gene expression were determined by real-time qPCR.

Results: Hemoglobin and hematocrit were significantly reduced in both ID groups compared to the C and PF groups. Similarly, animals in the both ID groups exhibited elevated steady-state levels of blood glucose and insulin, and significantly decreased levels of circulating cortisol compared to their respective PF controls. Serum triacyglycerols were only increased in ID animals consuming the AIN-76A diet. Hepatic gene expression analyses revealed a ~4- and 3-fold increase in the expression of glucokinase and pyruvate dehydrogenase kinase-4 mRNA, respectively, in the ID group on either diet compared to their respective PF counterparts. In contrast, the expression of lipogenic genes was significantly elevated in the AIN-76 ID group, while expression of these genes was unaffected by iron status in the AIN-93 ID group.

Conclusions: These results indicate that an impaired iron status is sufficient to alter glucose homeostasis, though alterations in lipid metabolism associated with ID are only observed in animals receiving the AIN-76A diet.

Keywords: Hyperglycemia, Lipogenesis, Insulin, Metabolism, Iron deficiency

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Background

Iron is an essential nutrient found in abundance in the earth's crust, yet iron deficiency remains the most common micronutrient deficiency in the world [1]. Symptoms of iron deficiency include weakness, fatigue, impaired immune function, and reduced cognitive function in children. In animal models, less well characterized responses to iron deficiency include alterations in lipid and glucose metabolism arising from decreased oxidative capacity which leads to a shift in preferential fuel utilization from fat to glucose [2-4]. Iron-deficient animals also display signs of disrupted metabolic homeostasis, including alterations in insulin signaling, as evidenced by hyperglycemia, hyperinsulinemia, and hyperlipidemia [2,5].

Determining the molecular mechanisms contributing to the hyperglycemic and hyperinsulinemic responses observed in response to dietary iron deficiency is made more complicated since many of the observations in glucose metabolism were noted prior to the 1993 American Institute of Nutrition (AIN) reformulation (AIN-93) of the AIN-76A laboratory animal diet, which is simply referred to as AIN-76 in the present study. The AIN-93 rodent diets were formulated to improve animal performance in experimental models with a major change being the substitution of cornstarch for sucrose because high dietary concentrations of sucrose were associated with several metabolic complications including hyperlipidemia, hyperinsulinemia, and fatty liver [6,7]. Therefore, it is difficult to discern the extent to which the metabolic consequences observed in previous studies noting disruptions in metabolic homeostasis were indeed an effect of iron deficiency alone, or rather the effect of dietary carbohydrate (i.e., sucrose) on glucose and lipid metabolism in iron-deficient animals.

Another factor complicating the investigation into the metabolic response to iron deficiency is that the severity of these consequences (i.e., hyperglycemia and hyperlipidemia) appears to be a graded response associated with a reduction in hemoglobin [8]. Hemoglobin levels indicative of anemia are associated with elevated plasma triacylglycerols (TAG) and glucose, though less severe reductions in hemoglobin are not as highly correlated with hyperlipidemia and hyperglycemia suggesting that a certain threshold exists in order to develop these potentially negative metabolic consequences [8-10]. In contrast, neither hyperglycemia nor hyperlipidemia were observed at varying levels of anemia in animals fed an AIN-93 based diet, although both glucose utilization and insulin responsiveness appeared to be enhanced [2]. More recently, two studies reported elevated serum glucose and TAG levels in severely iron-deficient (hemoglobin < 60 g/L) rodents fed an AIN-93 diet, but the underlying mechanisms contributing to these metabolic responses were not the primary focus of these investigations [11,12]. Thus, it remains unclear if these mixed observations from previous studies are more attributable to the severity of iron deficiency elicited in the animal model, or are instead the result of a carbohydrate-specific response to an iron-deficient diet.

The focus of the current study was to examine the extent to which an impaired iron status is associated with alterations in metabolic homeostasis and changes in hepatic lipogenic gene expression in rats fed iron-deficient diets based on either the AIN-76 or AIN-93 formulations. Animals in the iron-deficient groups, regardless of carbohydrate source, exhibited elevated levels of steady-state serum glucose and insulin. Interestingly, serum TAG and the relative abundance of mRNA encoding proteins responsible for regulating *de novo* lipogenesis in the liver was increased only in iron-deficient rats receiving the AIN-76 diet. The results presented herein support a model wherein alterations in glucose homeostasis observed in iron-deficient animals are independent of dietary carbohydrate whereas alterations in lipid metabolism appear to be dependent of dietary carbohydrate present in the AIN-76 diet. Thus, it is essential to consider the metabolic consequences of diets used to study the effects of micronutrient deficiencies in animal models.

Methods

Study design

Forty-eighty 21-d-old weanling male Sprague–Dawley (Harlan, IN) rats were housed individually at the Oklahoma State University (OSU) Laboratory Animal Research facility in a temperature- and humidity-controlled environment and maintained on a 12 h light:dark cycle with *ad libitum* access to deionized water. Upon arrival at the animal facility, rats were randomly assigned to either the AIN-76 (n=24) or AIN-93G (n=24) arm of the study (Table 1). Rats in each group were allowed *ad libitum* access to their respective control diet for 3 d prior to starting dietary treatments. After the acclimation period, rats in each group were assigned to one of three treatments (n=8/treatment) for 21 d: control (76-C or 93-C; 40 mg Fe/kg diet), iron-deficient (76-ID or 93-ID; ≤ 3 mg Fe/kg diet), or pair-fed (76-PF or 93-PF; each were fed their respective control (40 mg Fe/kg diet) diets at the level of intake of their ID counterparts). Commercially available powdered diets (76-C-TD.89300, 76-ID-TD.80396, 93-C-TD.94045, and 93-ID-TD.09564) were purchased from Harlan Teklad (Madison, WI). The primary differences between the AIN-76 and AIN-93 diets pertinent to the current study are shown in Table 1. Whereas cellulose was notably absent from the 93-ID in an effort to prevent iron contamination, the 93-C diet

Table 1 Composition of AIN-76 and AIN-93 diets[1]

	AIN-76 (g/kg)	AIN-93 (g/kg)
Casein	200	200
DL-Methionine	3	—
L-Cystine	—	3
Corn Starch	150	**447**
Maltodextrin	—	132
Sucrose	**550**	100
Soybean Oil	—	70
Corn Oil	50	—
Mineral Mix[2]	35	35
Vitamin Mix	10	10
Choline Bitartrate	2	2.5
TBHQ, antioxidant	—	0.014
Ethoxyquin	0.01	—

[1]The 93-C diet contained 397 g/kg corn starch with an added 50 g/kg cellulose. Cellulose was removed from the 93-ID and both AIN-76 diets to prevent contamination with additional iron.
[2]Mineral mixes were adjusted such that the ID diets contained ≤ 3 mg Fe/kg diet, and the C diets contained ~40 mg Fe/kg diet.

contained 50 g cellulose/kg diet. Previous studies have shown that this level of cellulose (5% w/w) does not adversely affect apparent absorption of iron [13]. Individual body weights and food intake were measured daily. After the 21 d experimental period, rats were anesthetized with a mixture of ketamine/xylazine and killed by exsanguination between 8:00 and 10:00 a.m. Food intake was not restricted prior to sacrifice. All animal handling and procedures were approved by the Institutional Animal Care and Use Committee at OSU.

Assessment of iron status

Whole blood was collected from the abdominal aorta into EDTA-coated tubes and sent to a commercial laboratory (Antech Diagnostics, Inc. Irvine, CA) for determination of hemoglobin and hematocrit. A sample of whole blood was also collected into serum tubes, allowed to clot, centrifuged at 800 x *g* for 20 min at 4°C to separate the serum, and then stored at −80°C until further analyses. Serum iron was determined using an ELAN 9000 ICP-Mass Spectrometer (PerkinElmer, Norwalk, CT). Microanalysis of non-heme iron in liver was determined as described by Rebouche *et al.* [14].

Metabolic indices

Serum glucose was measured using a glucose oxidase kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, except that reaction volumes were scaled down to a final volume of 1 mL. Serum was diluted 1:80 (v/v) so that results obtained were within the detectable range of the assay and standard curve. Serum insulin (Crystal Chem, Inc., Downers Grove, IL) and cortisol (R&D Systems, Minneapolis, MN) were measured by ELISA according to the manufacturer's instructions. Serum TAG levels were measured using a standard colorimetric assay based on the enzymatic hydrolysis of triacylglycerol to glycerol and free fatty acids by lipase (Sigma-Aldrich, St. Louis, MO). For all assays, serum samples with obvious signs of hemolysis were excluded from analyses.

Pathway-focused PCR array and qPCR

Changes in gene expression were analyzed by a pathway-focused Glucose Metabolism PCR array (SABiosciences, Valencia, CA). Briefly, total RNA was isolated from ~ 100 mg whole liver using STAT-60 (Teltest, Inc., Friendswood, TX). RNA concentration and integrity were determined using a Nanodrop spectrophotometer (Thermo Fischer Scientific, Middletown, VA) and agarose gel electrophoresis, respectively. Total RNA was then treated with DNase I, reverse-transcribed using SuperScript II (Invitrogen, Grand Island, NY), and brought to a final volume of 120 μL. The cDNA from individual animals was used as a template for the PCR array according to the array instructions using SYBR green chemistry on an ABI 7900HT system (Applied Biosystems, Grand Island, NY). Data were analyzed using SABiosciences RT^2 Profiler PCR Data Analysis software at http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php and were considered significant at > 1.3 fold change and $P < 0.05$. Relative quantitation for each gene was determined by normalizing to 4 housekeeping genes (RPLP1, HPRT1, RPL13A, and ACTB) comparing the ID and PF groups using the $2^{-\Delta\Delta Ct}$ method. For gene expression analysis by qPCR using SYBR green chemistry, cDNA was prepared as described above. Primers for qPCR were designed using Primer Express v 2.0 (Applied Biosystems) and validated if they met the following criteria: (1) single peak on dissociation curve and (2) amplification efficiency slope of −3.3 using titrated standard curve. Additionally, whenever possible, primers were designed such that the amplicon spanned at least one intron. Relative quantitation for each gene was determined using the $2^{-\Delta\Delta CT}$ method (Applied Biosystems User Bulletin #2) with Cyclophilin B (Cyclo) as the invariant control. Primer sequences used in these studies are listed in Additional file 1: Table S1.

Statistical analysis

Comparisons of body weight, hemoglobin, hematocrit, and array data within and between treatment groups were analyzed using one-way ANOVA followed by Least Significant Difference as the *post hoc* test using SPSS software version 17.0 (IBM-SPSS, Chicago, IL). Differences in gene expression between ID and PF groups within each diet were determined using a Student's

t-test. All tests were conducted at the 95% confidence interval and presented as means ± SEM.

Results

Body weight and food intake

Consistent with previous observations, rats in the ID groups consumed significantly less diet and weighed significantly less than rats in the C groups regardless of diet formulation (Figure 1) [15,16]. At the end of the dietary treatment period, rats in the 76-ID group consumed 13% less diet and weighed 10% less than the 76-C group, whereas rats in the 93-ID group consumed 7% less diet and weighed 5% less than the 93-C group. There were no differences in final body weight between rats in the PF and ID groups on either diet (Figure 1). Interestingly, rats receiving either the 76-C or 76-ID diets consumed significantly less diet than those receiving the 93-C or 93-ID diet, respectively.

Iron status

Importantly, only the rats in the ID groups exhibited signs of anemia as determined by hemoglobin and hematocrit (Figure 2). Regardless of diet, hemoglobin levels were decreased by ~38% in the ID groups compared to C and PF groups (Figure 2A). Similarly, rats in the 76-ID and 93-ID groups exhibited a ~37% decline in hematocrit values compared to the C and PF groups (Figure 2B). Because of the similarities in hemoglobin and hematocrit between the C and PF rats within each diet group, the remaining comparisons were made between the ID and PF groups for each diet in order to attribute any observed biological changes to a diminished iron status rather than a decrease in total nutrient intake. Lastly, serum iron and non-heme liver iron was significantly lower in the ID groups compared to the PF groups for both diets (Figure 3A-B).

It is of note that serum iron levels in the 93-PF group were significantly lower than the 76-PF group (Figure 3A). Each diet was sent to an independent laboratory (N-P Analytical Laboratories, St. Louis, MO) for analysis of iron content. The iron content of the 76-C and 93-C diets was 44 mg/kg and 41 mg/kg diet, respectively. Iron content of the 76-ID and 93-ID diets was 3 mg/kg and 2 mg/kg diet, respectively. Thus, when average daily intake of iron is compared by diet (i.e., AIN-76 *vs.* AIN-93), rats receiving the AIN-93 diet consumed less iron those consuming the AIN-76 diet, regardless of iron level in the diet (607.2 ± 25 μg/day for 76-PF *vs.* 590.8 ± 25 μg/day for 93-PF and 47.6 ± 2 μg/day for 76-ID *vs.* 31.2 ± 1 μg/day for 93-ID, *data not shown.*) Despite these differences, animals in the PF groups met the recommended guidelines for iron intake of at least 525 μg Fe/day (based on an intake of 15 g diet/day on a diet containing 35 mg Fe/kg diet) [17]. Interestingly, non-heme liver iron was not different between the 76-PF and 93-PF groups (Figure 3B), and importantly serum iron and non-heme liver iron did not differ between the 76-ID and 93-ID groups (Figure 3A-B).

Steady-State serum levels of glucose, insulin, and triacylglycerols

Serum glucose levels were higher than normal in all groups, but were expected as a result of the method of

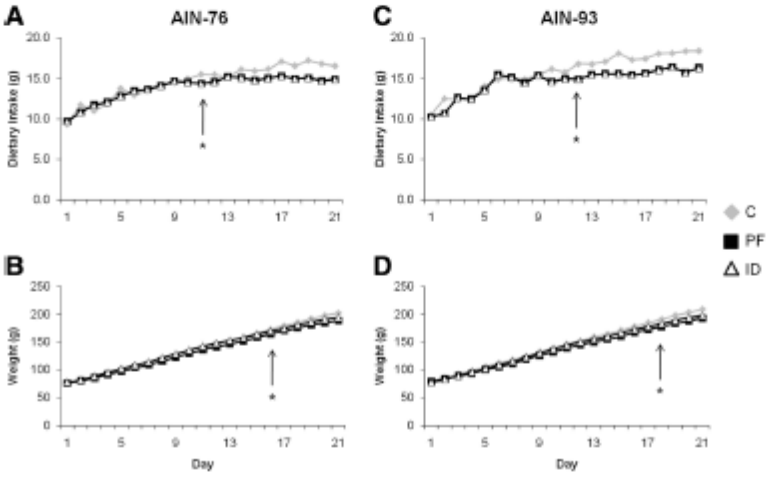

Figure 1 Body weights and food intake were monitored throughout the 21 d experimental period. Both the ID and PF groups gained significantly less weight regardless of diet. *Indicates the day at which differences in food intake and weight gain became statistically significant, $p < 0.05$.

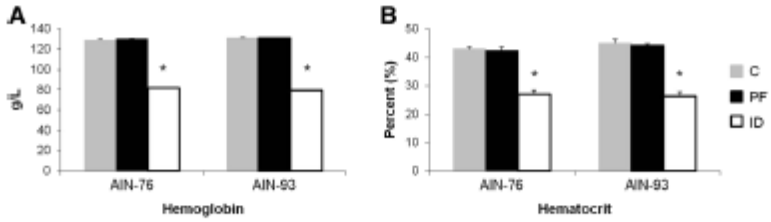

Figure 2 Hematologic values indicate that only the ID animals in each group were anemic. Levels of hemoglobin and hematocrit did not differ between AIN-76 and AIN-93 diets. Values are means ± SEM (n = 8). *Indicates statistical significances between groups, $p < 0.05$.

anesthesia used in this study [18,19]. Although not statistically significant (P=0.06), serum glucose in the 76-ID group tended to be higher when compared to the 76-PF group (Figure 4A). In animals receiving the AIN-93 diet, serum glucose of rats in the ID group was significantly higher than those in the 93-PF group (P<0.05) (Figure 4A). Serum insulin levels were increased 50% and 100% in the 76-ID and 93-ID groups, respectively, compared to their corresponding PF groups (P<0.05) (Figure 4B). In order to determine if elevated glucose levels were the result of increased circulating cortisol, serum cortisol was determined. Compared to their respective PF groups, serum cortisol levels were significantly decreased in the 76-ID and 93-ID groups (P<0.05) (Figure 4C). Intriguingly, dietary iron deficiency was only associated with elevated serum levels of TAG in rats consuming the AIN-76 diet (P<0.05) (Figure 4D).

Alterations in hepatic gene expression

Using a pathway-focused PCR array approach in addition to traditional qPCR, the expression of genes involved in glucose and lipid metabolism was assessed in the livers of ID and PF animals. Relative to the PF groups, more numerous and significant changes in gene expression were detected in the 76-ID group than the 93-ID group in the PCR array (Table 2). In terms of glucose homeostasis, the most notable change in gene expression that was determined by the glucose metabolism PCR array was a ~3.5-fold increase in glucokinase (*Gck*) mRNA expression in both ID groups (Table 2). In contrast, ATP-citrate lyase (*Acly*) increased 2.9-fold in the 76-ID group compared to the 76-PF group, though similar changes were not evident in rats receiving AIN-93-based diets (Table 2). The alterations in gene expression of both *Gck* and *Acly* determined by the PCR array were further validated and confirmed by qPCR (Figure 5A-B).

Taking a more biased approach to assess iron- and/or diet-dependent changes in gene expression, the hepatic expression of genes involved in glucose, lipid, and iron metabolism was also examined by qPCR. The mRNA abundance of pyruvate dehydrogenase kinase-4 (*Pdk4*) was increased ~3- and 2-fold in the 76-ID and 93-ID groups, respectively (Figure 5A-B). Similar to the changes in *Acly* gene expression observed in the 76-ID group, the expression of lipogenic genes such as sterol regulatory binding protein-1c (*Srebp1c*) and fatty acid synthase (*Fas*) were also significantly increased in the livers of the 76-ID animals compared to the 76-PF animals (Figure 5A). Compared to the 93-PF group, expression of *Srebp1c* and *Fas* was not significantly different in the 93-ID group (Figure 5B). Interestingly, the expression of stearoyl CoA desaturase 1 (*Scd1*) mRNA, the rate-limiting enzyme involved in the synthesis of

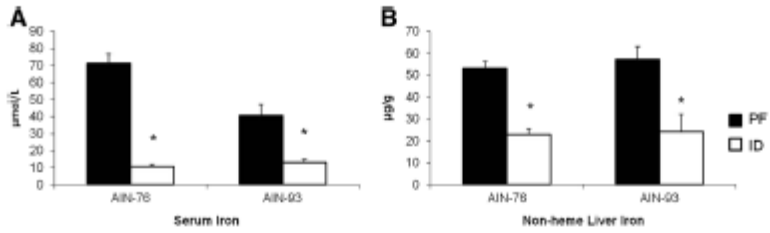

Figure 3 Degrees of iron deficiency did not differ between AIN-76 and AIN-93 diets. Serum iron and non-heme liver iron were reduced to similar degrees in ID animals fed either an AIN-76 or AIN-93 diet. Values are means ± SEM (n = 8). *Indicates statistical significance between groups, $p < 0.05$.

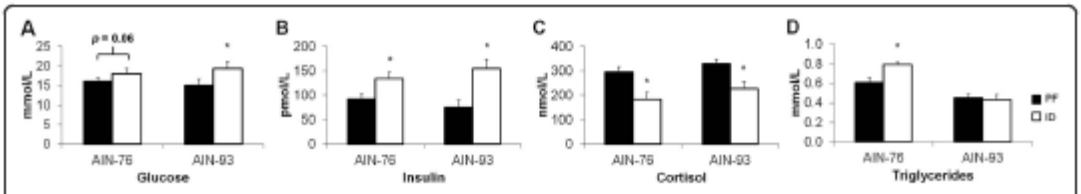

Figure 4 Serum levels of glucose and insulin were significantly increased, while cortisol levels were significantly decreased in the ID groups. ID resulted in significantly increased serum triglycerides in rats consuming the AIN-76 diet, but did not have an effect on rats consuming the AIN-93 diet. Values are given as means ± SEM (n=4-8). *Indicates statistical difference between ID and PF animals within each diet group, $p < 0.05$.

monounsaturated fatty acids for subsequent incorporation into TAG, was significantly decreased (~60% reduction) in the 76-ID group compared to the 76-PF group (Figure 5A). Similar results were obtained for the 93-ID group, though when compared to the 93-PF group, the results did not reach the level of statistical significance (Figure 5B). Consistent with previous findings of diminished β-oxidation activity in ID, there was significant reduction (70 – 80%) in the gene expression of carnitine palmitoyltransferase (*Cpt1*) in both ID groups regardless of diet (Figure 5A-B) [9,10,20]. Lastly, to further confirm that the livers were "sensing" iron deficiency in animals receiving an iron-deficient diet, the mRNA abundance of the iron uptake protein transferrin receptor 1 (*Tfr1*) and the iron-sensing peptide hormone hepcidin (*Hamp1*) was assessed. As expected, both the 76-ID and 93-ID groups exhibited an increased abundance (4 – 7-fold) of *Tfr1* mRNA compared to their respective PF groups (Figure 5A-B). Expression of *Hamp1* mRNA was significantly repressed (> 99% reduction) in both ID groups compared to their PF counterparts (Figure 5A-B).

Table 2 Relative fold-change in mRNA abundance of genes involved in glucose metabolism[1]

Gene Name	Gene Symbol	AIN-76	AIN-93
Glucokinase	Gck	+3.4	+3.9
ATP-citrate lyase	Acly	+2.9	NC
Aconitase 1 (cytosolic; IRP1)	Aco1	+1.3	+1.6
Pyruvate dehydrogenase (lipoamide) beta	Pdhb	+1.5	NC
Phosphoglucomutase 3	Pgm3	+1.5	NC
Ribose 5-phosphate isomerase A	Rpia	+1.5	+1.4
Glycogen synthase 2 (liver specific)	Gys2	−1.3	−1.6
2,3-bisphosphoglycerate mutase	Bpgm	−1.3	−1.4

[1]Values in each column indicate statistically significant relative fold-changes in mRNA abundance in each ID group compared to their respective PF group. NC is no change. Statistical significance was defined as fold-change ≥ 1.3 and $p < 0.05$.

Discussion

In the current study, despite a severe dietary iron restriction, only a moderate degree of anemia (hemoglobin ~ 80 g/L) was elicited. Interestingly, even a moderate induction of iron deficiency appears to contribute to elevations in both steady-state levels of serum glucose and insulin regardless of basal diet formulation. Others have postulated that an increase in serum glucose may be due, at least in part, to an elevation of cortisol observed in severely anemic rats [21,22]. Interestingly, the relative decrease in cortisol in the ID groups in the current study suggests that other mechanisms may be responsible for the presence of hyperglycemia. In fact, the results of this study are in agreement with those obtained in a human study wherein patients with severe iron deficiency exhibited reduced cortisol secretion [23]. In addition to the relative hyperglycemia, a relative hyperinsulinemia was observed in the ID animals in both groups as well. These metabolic adaptations presumably occur as a compensatory means as ID animals preferentially utilize glucose, rather than fat, as a metabolic substrate for peripheral tissues as a result of decreased hemoglobin levels and subsequent decreased oxidative capacity [4,9,10]. Thus, blood glucose levels likely remain elevated to ensure that adequate fuel substrate is available for energy production, and insulin levels may remain elevated to facilitate the entry of glucose into insulin-dependent tissues.

In an effort then to further interrogate the underlying metabolic changes that occur with iron deficiency, we examined the hepatic expression of 84 genes involved in maintenance of glucose homeostasis. The level of significance (fold change ≥ 1.3), while small, was considered significant as previous studies have shown that dietary intervention(s) tend to elicit somewhat modest, yet biologically meaningful, transcriptional responses [24,25]. The ID rats in each group exhibited modest, but significant alterations in the expression of genes representative of glucose metabolism. Notable changes in gene expression include those genes associated with metabolic pathways including both glycolysis and gluconeogenesis and are in agreement with the findings of others [2,10,26].

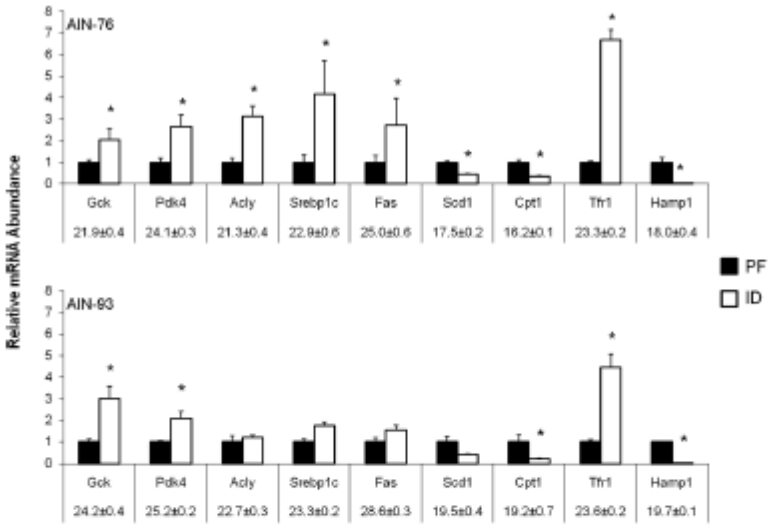

Figure 5 Iron deficiency significantly affects the hepatic expression of mRNA involved in fat metabolism (*Fas*, *Acly*, *Scd1*) and insulin signaling (*Pdk4* and *Srebp1c*). The ID group also exhibited in significantly increased expression of *Tfr1* and significantly decreased expression of the iron sensor hepcidin (*Hamp1*). mRNA levels were normalized to Cyclophilin B (*Cyclo*) mRNA as the invariant control. Numbers beneath gene names indicate C_T value ± SEM obtained for the PF group. *Cyclo* Mean C_T = 18.9 for both PF groups. Values are given as means ± SEM (n=8). *Indicates statistical difference between ID and PF animals within each diet group, $p < 0.05$.

The significant increase in *Gck* expression is likely due to the relative increase in circulating insulin levels observed in the ID groups, as insulin is a known inducer of hepatic *Gck* mRNA expression [27,28]. Increased expression of *Gck* could potentially be very important as ID animals have been shown to have an increased reliance on glucose as a metabolic substrate, and *Gck* is able to rapidly increase the rate of glucose phosphorylation in the liver in response to the elevations in blood glucose levels [27]. Furthermore, as Gck catalyzes the first step in hepatic glucose utilization it can contribute multiple pathways including glycogen synthesis, glycolysis, and *de novo* lipogenesis which could explain the enhanced glucose utilization and hyperlipidemia reported in response to dietary ID [2,10,26,29,30].

The significant increase in *Gck* mRNA expression, presumably in response to the elevated insulin levels, suggests that insulin signaling in ID animals is at least partially intact. However, previous observations suggest that alterations in metabolic gene expression are indicative of an impaired hepatic insulin response wherein ID animals exhibited a form of mixed insulin resistance [5]. Under normal conditions (e.g., iron sufficiency) when insulin levels are elevated in a fed state, insulin acts both to repress gluconeogenic gene expression and to simultaneously activate lipogenic gene expression [31,32]. In contrast, chronic hyperinsulinemia contributes to a combination of hepatic insulin resistance in which the insulin-dependent activation of lipogenic gene expression remains intact, but gluconeogenic gene expression is inadequately repressed [5,30,33]. In this model of mixed insulin resistance, insulin acts through the mammalian target of rapamycin complex 1 to activate lipogenesis via a *Srebp1c*-dependent increase in lipogenic gene expression, whereas insulin-induced phosphorylation of the transcription factor forkhead box protein O1 is diminished such that gluconeogenic gene expression remains inappropriately active [33]. Thus, mixed insulin resistance remains a candidate mechanism explaining the relative hyperglycemia and hyperlipidemia reported in ID animals. Despite changes in hepatic insulin signaling, peripheral tissue insulin sensitivity as assessed by glucose clearance appears to be enhanced with ID [2,4]. The extent to which there are tissue-specific differences in insulin signaling in response to iron deficiency warrants additional investigation.

To further investigate potential factors contributing to hypertriacylglycerolemia that has been reported in iron-deficient animals, changes in hepatic lipogenic gene expression were assessed in the PF and ID groups on both diets. In fact, it was the finding of increased lipogenic gene expression in iron-deficient animals consuming an AIN-76-based diet that stimulated the comparison of

the AIN-76 and AIN-93 diets [5]. As early as 1982 it was speculated that causes of metabolic complications observed in animals consuming an AIN-76 diet were related to sucrose, the primary carbohydrate source in the AIN-76 formulation [34]. Not surprisingly, basal levels of the lipogenic genes *Acly* and *Fas* were higher in the 76-PF group compared to the 93-PF group, though the expression of the lipogenic master transcriptional regulator *Srebp1c* remained largely unchanged. Nonetheless, compared to the PF group, lipogenic gene expression was further significantly enhanced only in animals consuming an iron-deficient AIN-76 diet. Elevated levels of TAG in the 76-ID group suggest a functional consequence of enhanced lipogenic gene expression resulting is *de novo* fatty acid synthesis and packaging into TAG destined for secretion from the liver. Although the levels of liver TAG were not determined in the present study, taken together these data provide compelling evidence that consumption of an iron-deficient yet high-sucrose diet may result in more severe metabolic complications leading to hyperglycemia, hyperinsulinemia, and hyperlipidemia.

Despite the differences observed in lipogenic gene expression and serum TAG between diets and consistent with the findings of others that β-oxidation is decreased in response to dietary ID, the mRNA expression of *Cpt1* was found to be significantly reduced in both the 76- and 93-ID groups. The expression of the iron-containing enzyme *Scd1* was also decreased in the 76-ID group, with the same trend being observed in the 93-ID group. Importantly, expression of the mRNA encoding for the iron uptake protein *Tfr1* was significantly increased while expression of the iron sensor *Hamp1* was significantly decreased in both ID groups. Furthermore, the relative abundance of both *Tfr1* and *Hamp1* mRNA was not different between diets for either the PF or ID animals indicating a similar response to dietary ID, regardless of basal diet. No differences in serum iron or non-heme liver iron between the ID groups on either diet also show that a similar degree of iron deficiency was induced in both ID groups. The moderate level of iron deficiency achieved in this study may serve as an explanation for the differences noted in lipogenic gene expression and serum TAG. Previous work investigating the metabolic lipid response to varying degrees of iron deficiency on an AIN-93 based diet only observed a significant increase in serum TAG at hemoglobin ≤ 66 g/L, a level which is consistent with data suggesting that the severity of metabolic responses to iron deficiency is most highly associated with reductions in hemoglobin [2,4,8,12,35]. Interestingly, despite a more modest induction of iron deficiency for the current study (hemoglobin = 80 g/L), relative elevations in serum glucose and insulin levels were still observed in both the 76-ID and 93-ID groups.

Our findings support the work of others that have clearly demonstrated that even a moderate induction of iron deficiency is sufficient to disrupt normal glucose homeostasis in rodents [2,3,8-10,12,26]. To date, relatively little evidence is available for humans, but an association between ID and elevated hemoglobin A1C (HbA1c) levels has been observed [36]. Indeed, as iron status is improved, levels of HbA1c return to more normal levels [36]. Interestingly, despite what some attribute to so-called over-nutrition, ID is also commonly observed in overweight and obese individuals, a population in which metabolic homeostasis is often disrupted [37-39]. The potential for ID to contribute to or exacerbate conditions wherein normal glucose and fat metabolism are already impaired will be the focus of future investigations.

Currently, the specific mechanisms contributing to iron deficient-induced hyperglycemia remain elusive, but the findings presented herein support the hypothesis that a depletion of iron status, regardless of dietary carbohydrate source, is sufficient to disrupt systemic glucose homeostasis in a weanling rat model of iron deficiency. Intriguingly, enhanced expression of the lipogenic genes *Srebp1c*, *Acly*, and *Fas* was only observed in the ID-76 group. The extent to which these results are suggestive of an iron-level by carbohydrate (i.e., sucrose) interaction remains unknown and is the focus of future studies. To this end, it will also be important to closely examine the changes in nutrient sensing and insulin signaling that occur in response to dietary iron deficiency across different tissues such as the liver and skeletal muscle. The significant increase in *Gck* expression in both ID groups provides some of the first insight into the means by which glucose uptake and utilization is altered in response to iron deficiency [2,9,26]. Future work should focus on the biological significance of this and the other somewhat modest transcriptional changes that occurred with particular attention to the allocation of glucose to various pathways.

Conclusions

Alterations in serum glucose and insulin observed in ID animals are likely to result from an impaired iron status, rather than the altered utilization of dietary carbohydrate. In contrast, the alterations observed in lipid metabolism (e.g., lipogenic gene expression and serum TAG) in response to ID may be explained by metabolic partitioning of the excess sucrose (relative to AIN-93) present in the AIN-76 diet.

Additional file

Additional file 1: Table S1. Gene Symbols, NCBI Accession Numbers, and Primer Sequences for qPCR.

Abbreviations

AIN: American Institute of Nutrition; C: Control; ID: Iron deficient; PF: Pair fed; TAG: Triacylglycerol; Gck: Glucokinase; Acly: ATP-citrate lyase; Aco1: Aconitase 1; Phdh: Pyruvate dehydrogenase (lipoamide) beta; Pgm3: Phosphoglucomutase 3; Rpia: Ribose 5-phosphate isomerase A; Gys1: Glycogen synthase 1; Gys2: Glycogen synthase 2; Bpgm: 2,3-bisphosphoglycerate mutase; Pdk4: Pyruvate dehydrogenase kinase-4; Cpt1: Carnitine palmitoyltransferase; Fas: Fatty acid synthase; Scd1: Stearoyl-CoA desaturase; Srebp1c: Sterol regulatory element binding protein-1c; Tfr1: Transferrin receptor 1; Hamp1: Hepcidin.

Competing interest

McKale R. Davis, Kristen K. Hester, Krista M. Shawron, Edralin A. Lucas, Brenda J. Smith, and Stephen L. Clarke have no competing interests.

Authors' contributions

MRD and SLC designed the research. MRD, KKH, KMS and SLC conducted the research. MRD, KKH, EAL, BJS, and SLC collected and analyzed data. MRD, BJS, EAL, and SLC wrote the paper. All authors read and approved the final manuscript.

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References

1. McLean E, Cogswell M, Egli I, Wojdyla D, de Benoist B: **Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005.** *Public Health Nutr* 2009, 12(4):444–454.
2. Borel MJ, Beard JL, Farrell PA: **Hepatic glucose production and insulin sensitivity and responsiveness in iron-deficient anemic rats.** *Am J Physiol* 1993, 264:E380–E390.
3. Davies KJ, Donovan CM, Refino CJ, Brooks GA, Packer L, Dallman PR: **Distinguishing effects of anemia and muscle iron deficiency on exercise bioenergetics in the rat.** *Am J Physiol* 1984, 246:E535–E543.
4. Farrell PA, Beard JL, Druckenmiller M: **Increased insulin sensitivity in iron-deficient rats.** *J Nutr* 1988, 118:1104–1109.
5. Davis MR, Rendina E, Peterson SK, Lucas EA, Smith BJ, Clarke SL: **Enhanced expression of lipogenic genes may contribute to hyperglycemia and alterations in plasma lipids in response to dietary iron deficiency.** *Genes Nutr* 2012, 7:415–425.
6. Reeves PG: **Components of the AIN-93 diets as improvements in the AIN-76A diet.** *J Nutr* 1997, 127:838S–841S.
7. Sanchez-Lozada LG, Mu W, Roncal C, Sautin YY, Abdelmalek M, Reungjui S, Le M, Nakagawa T, Lan HY, Yu X, Johnson RJ: **Comparison of free fructose and glucose to sucrose in the ability to cause fatty liver.** *Eur J Nutr* 2010, 49:1–9.
8. Borel MJ, Smith SH, Brigham DE, Beard JL: **The impact of varying degrees of iron nutriture on several functional consequences of iron deficiency in rats.** *J Nutr* 1991, 121:729–736.
9. Brooks GA, Henderson SA, Dallman PR: **Increased glucose dependence in resting, iron-deficient rats.** *Am J Physiol* 1987, 253:E461–E466.
10. Linderman JK, Brooks GA, Rodriguez RE, Dallman PR: **Maintenance of euglycemia is impaired in gluconeogenesis-inhibited iron-deficient rats at rest and during exercise.** *J Nutr* 1994, 124:2131–2138.
11. Yamagishi H, Okazaki H, Shimizu M, Izawa T, Komabayashi T: **Relationships among serum triacylglycerol, fat pad weight, and lipolysis in iron-deficient rats.** *J Nutr Biochem* 2000, 11:455–460.
12. Yamagishi HaK T: **Alteration of glucose metabolism and increased fructosamine in iron-deficiency anemic rats.** *Nutr Res* 2003, 23:1547–1553.
13. Gordon DT, Beach-Williford C, Ellersieck MR: **The action of cellulose on the intestinal mucosa and element absorption by the rat.** *J Nutr* 1983, 113:2545–2556.
14. Rebouche CJ, Wilcox CL, Widness JA: **Microanalysis of non-heme iron in animal tissues.** *J Biochem Biophys Methods* 2004, 58:239–251.
15. Chen OS, Schalinske KL, Eisenstein RS: **Dietary iron intake modulates the activity of iron regulatory proteins and the abundance of ferritin and mitochondrial aconitase in rat liver.** *J Nutr* 1997, 127:238–248.
16. Katsumata S, Katsumata-Tsuboi R, Uehara M, Suzuki K: **Severe iron deficiency decreases both bone formation and bone resorption in rats.** *J Nutr* 2009, 139:238–243.
17. Subcommittee on Laboratory Animal Nutrition CoAN, Board on Agriculture, National Research Council: *Nutrient Requirements of Laboratory Animals.* 4th edition. Washington D.C.: The National Academies Press; 1995.
18. Brown ET, Umino Y, Loi T, Solessio E, Barlow R: **Anesthesia can cause sustained hyperglycemia in C57/BL6J mice.** *Vis Neurosci* 2005, 22:615–618.
19. Pomplun D, Mohlig M, Spranger J, Pfeiffer AF, Ristow M: **Elevation of blood glucose following anaesthetic treatment in C57BL/6 mice.** *Horm Metab Res* 2004, 36:67–69.
20. Gregg SG, Mazzeo RS, Budinger TF, Brooks GA: **Acute anemia increases lactate production and decreases clearance during exercise.** *J Appl Physiol* 1989, 67:756–764.
21. Campos MS, Barrionuevo M, Alferez MJ, Gomez-Ayala AE, Rodriguez-Matas MC, Lopez Aliaga I, Lisbona F: **Interactions among iron, calcium, phosphorus and magnesium in the nutritionally iron-deficient rat.** *Exp Physiol* 1998, 83:771–781.
22. Dallman PR, Refino CA, Dallman MF: **The pituitary-adrenal response to stress in the iron-deficient rat.** *J Nutr* 1984, 114:1747–1753.
23. Saad MJ, Morais SL, Saad ST: **Reduced cortisol secretion in patients with iron deficiency.** *Ann Nutr Metab* 1991, 35:111–115.
24. Gaj S, Eijssen L, Mensink RP, Evelo CT: **Validating nutrient-related gene expression changes from microarrays using RT(2) PCR-arrays.** *Genes Nutr* 2008, 3:153–157.
25. Jae-Eun Pie Y-RK, In-Kyoung K, Sang-Hui S, Seung Ho L, Hee Ra L, Young Y, Ji Tae C, Jong Pil Y, Moonlu O, Seung Yong H, Meyoung-Kon K: **Correlation between nutrition intake and gene expression profiles in children with asthma.** *Mol Cell Toxicol* 2010, 6:313–319.
26. Henderson SA, Dallman PR, Brooks GA: **Glucose turnover and oxidation are increased in the iron-deficient anemic rat.** *Am J Physiol* 1986, 250:E414–E421.
27. Iynedjian PB: **Molecular physiology of mammalian glucokinase.** *Cell Mol Life Sci* 2009, 66:27–42.
28. Iynedjian PB, Marie S, Gjinovci A, Genin B, Deng SP, Buhler L, Morel P, Mentha G: **Glucokinase and cytosolic phosphoenolpyruvate carboxykinase (GTP) in the human liver. Regulation of gene expression in cultured hepatocytes.** *J Clin Invest* 1995, 95:1966–1973.
29. Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Ruegg MA, Hall MN: **Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c.** *Cell Metab* 2012, 15:725–738.
30. Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, Goldstein JL: **Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice.** *Mol Cell* 2000, 6:77–86.
31. Accili D, Arden KC: **FoxOs at the crossroads of cellular metabolism, differentiation, and transformation.** *Cell* 2004, 117:421–426.
32. Horton JD, Goldstein JL, Brown MS: **SREBPs: transcriptional mediators of lipid homeostasis.** *Cold Spring Harb Symp Quant Biol* 2002, 67:491–498.
33. Li S, Brown MS, Goldstein JL: **Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis.** *Proc Natl Acad Sci U S A* 2010, 107:3441–3446.
34. Medinsky MA, Popp JA, Hamm TE, Dent JG: **Development of hepatic lesions in male Fischer-344 rats fed AIN-76A purified diet.** *Toxicol Appl Pharmacol* 1982, 62:111–120.
35. Stangl GI, Kirchgessner M: **Different degrees of moderate iron deficiency modulate lipid metabolism of rats.** *Lipids* 1998, 33:889–895.
36. Coban E, Ozdogan M, Timuragaoglu A: **Effect of iron deficiency anemia on the levels of hemoglobin A1c in nondiabetic patients.** *Acta Haematol* 2004, 112:126–128.

37. Lecube A, Carrera A, Losada E, Hernandez C, Simo R, Mesa J: **Iron deficiency in obese postmenopausal women.** *Obesity (Silver Spring)* 2006, 14:1724–1730.
38. Pinhas-Hamiel O, Newfield RS, Koren I, Agmon A, Lilos P, Phillip M: **Greater prevalence of iron deficiency in overweight and obese children and adolescents.** *Int J Obes Relat Metab Disord* 2003, 27:416–418.
39. Yanoff LB, Menzie CM, Denkinger B, Sebring NG, McHugh T, Remaley AT, Yanovski JA: **Inflammation and iron deficiency in the hypoferremia of obesity.** *Int J Obes (Lond)* 2007, 31:1412–1419.

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CHAPTER V

MIR-210 IS A POTENTIAL HOMEORHETIC REGULATOR OF IRON AND OXYGEN SIGNALING PATHWAYS

Note: The following manuscript is a work in progress that is being considered for submission to the Journal of Molecular Cell Biology

Abstract

Iron is an essential nutrient critical for oxygen transport, DNA synthesis, ATP generation, and cellular proliferation. At the molecular level, iron deficiency (ID) elicits a cascade of cellular events aimed at conserving iron for the maintenance of these life-preserving functions, but tissue-specific responses and metabolic adaptations to ID are not fully understood. Recently, small regulatory RNA molecules called miRNAs have been identified as an important mechanism for regulating various cellular processes. Therefore, we sought to determine the extent to which expression of miRNA is regulated in response to dietary ID and to examine their potential regulatory capacity in the adaptive response to ID. Utilizing a genome-wide miRNA microarray and a low-density PCR array we identified miR-210 as an iron responsive miRNA. *In silico* prediction programs and reporter assays were then used to predict and validate the iron containing heme-protein cytoglobin as a miR-210 target. Examination of the iron-dependent response of the established miR-210 target, Iscu, and the newly established target, cytoglobin provide new insight into the homeostatic regulation of iron metabolism by miRNA.

Introduction

Iron is an essential, yet potentially toxic nutrient, thus iron homeostasis must be tightly regulated to ensure adequacy and prevent overload. Iron balance is maintained by two regulatory systems that function to coordinate iron homeostasis at both systemic and cellular levels. Hepcidin is a key iron regulatory peptide hormone primarily responsible for coordinating systemic iron homeostasis by inversely affecting the rate of intestinal absorption and/or iron release from macrophages cells based on body iron stores [1]. At the cellular level, two iron regulatory proteins (IRP1 and IRP2) coordinate iron homeostasis by "sensing" intracellular iron status and accordingly coordinating the uptake, storage, and utilization of iron through high affinity RNA binding to iron responsive elements (IRE) found in mRNA encoding genes involved in iron metabolism [2]. Despite exhibiting similar regulatory functions, the IRP1 and IRP2 themselves are regulated quite differently. While IRP2 is targeted for proteosomal degradation via iron- and oxygen-dependent prolyl hydroxylase, the functional role of IRP1 is largely dependent on the presence of an iron-sulfur (Fe-S) cluster [3]. Whereas under iron deficient conditions the Fe-S cluster is removed and IRP1 functions as an RNA binding protein to restore iron homeostasis, under iron replete conditions, the Fe-S cluster is stabilized and IRP1 functions as the cytosolic isoform of aconitase (c-acon) [2, 4].

Proteins containing Fe-S clusters are found in virtually all organisms, and within multiple cellular compartments including the mitochondria, cytosol, and nucleus. Fe-S clusters in proteins act as cofactors that are essential for numerous biologic processes including maintenance of iron homeostasis, mitochondrial respiration, electron transfer, metabolism, and many other regulatory processes [5]. Skeletal muscle is severely

affected by iron deficiency due to the loss of Fe-S proteins, which are essential for muscle respiratory function as critical components of the ETC [6, 7]. Interestingly however, while the skeletal muscle is severely affected by iron deficiency, the liver appears to be relatively resistant to changes in iron status [8, 9]. For example, c-acon activity is unchanged in the liver, but significantly decreased in the muscles of iron deficient rats [8, 10]. Furthermore, significant reductions in mitochondrial enzyme activity, Fe-S enzyme content, and nitrogen fixation 1 homolog (Nfs1 or IscS) protein abundance have been reported in skeletal muscle in response to dietary iron deficiency, but are largely unaffected in the liver [8, 11, 12]. Given the importance of Fe-S proteins in the regulation of iron homeostasis (IRP1) and energy production (i.e., mitochondrial aconitase; m-acon), it is of interest to identify and elucidate regulatory factors involved in the formation and maintenance of Fe-S clusters, particularly in response to iron deficiency.

In addition to the consequences associated with the loss of Fe-S cluster protein activity, a major reason for the side effects observed with iron deficiency is due to the essentiality of iron for the biosynthesis of heme, which as the primary component of hemoglobin makes oxygen transport possible. Recent findings have expanded the physiologic roles of heme even further as a potential regulator of mRNA stability and degradation via the critical role it has been shown to play in microRNA (miRNA) processing [13, 14]. miRNA are a class of noncoding RNA approximately 22 nucleotides (nt) long that are now predicted to regulate as much as 60% of all protein-coding genes, and as such, are anticipated to participate in nearly every biological process within the cell [15, 16]. Provided the increasing roles for miRNA to fine-tune gene expression and

coordinate cellular functions, it is reasonable to speculate that nutrient availability or nutritional status might affect miRNA expression in an effort to maintain nutrient homeostasis. Thus, in addition to understanding the potential impact of iron status on miRNA processing, it is of interest to determine the extent to which miRNA contribute to the regulation of iron metabolism.

In fact, the liver-specific miR-122 has already been implicated as a contributory factor in systemic iron homeostasis as depletion of miR-122 in mice resulted in decreased plasma and liver iron, and mildly impaired hematopoiesis by targeting two transcriptional activators of hepcidin, the key systemic iron regulatory hormone [17]. Furthermore, the oxygen sensitive miR-210 is an established regulator of the Fe-S cluster assembly proteins, iron-sulfur cluster scaffold homolog (Iscu) under hypoxic conditions in cultured cells, and thus could play an important role in the control of iron utilization during certain environmental stimuli [18, 19]. These findings suggest that miRNA may be key regulators in many facets of human iron homeostasis, but to date an investigation into the extent to which *dietary* iron influences miRNA expression or regulation in a whole-animal has not been fully described. This evidence for the role of miRNA in modulating iron homeostasis is underscored by the fact that miRNA processing is, at least in part, a heme-dependent process [13, 14].

Thus, our primary objectives were to determine the extent to which expression of miRNA is regulated in response to ID and to characterize the impact of miRNA expression on potential regulatory targets involved in iron metabolism. In this study we have shown that miR-210 expression can be regulated independently of hypoxia as its expression is also increased in response to dietary iron deficiency. Furthermore, we have

identified and validated the hemoprotein cytoglobin as a newly established miR-210 target. Lastly, we have demonstrated that tissue-specific responses to iron deficiency could potentially be coordinated via miRNA-dependent regulation. This research provides novel insight as to how miRNA contribute to the cellular adaptation to iron deficiency and the molecular coordination of iron homeostasis.

Results

Iron deficient (ID) animals exhibited a 40% reduction in hemoglobin and a 38% reduction in hematocrit after 21 d on the low iron diet compared to their pair-fed (PF) controls (Table 1). Serum iron and liver iron were also reduced 85% and 63%, respectively in response to an ID diet (Table 1). The reduction in iron status corresponded with a 1.5-fold and 2-fold increase in spontaneous hepatic IRP1 and IRP2 RNA binding activity, respectively (Figure 1A and B). Intriguingly, soleus IRP2 RNA binding activity was also increased slightly more than 2-fold, but soleus IRP1 RNA binding activity was increased nearly 4-fold (Figure 1 E and F). Also of note, was the lack of change in total IRP1 RNA (Figure 1C and D) binding activity, but a significant, albeit small, reduction in total soleus IRP1 RNA binding activity (Figures 1G and H).

Table 1: Hematologic indices of iron status

Indices of Iron Status	PF	ID
Hemoglobin (d/dL)	13.3 ± 0.1	8.0 ± 0.2
Hematocrit (%)	43.8 ± 0.5	27.5 ± 1.6
Serum Iron (μM)	71.4 ± 5.2	10.8 ± 1.4
Liver Iron (μg/g)	245.9 ± 30.1	92.6 ± 16.0

Values are means ± SEM, n = 8/group
*Indicates statistical significance from between PF and ID groups ($P < 0.05$)

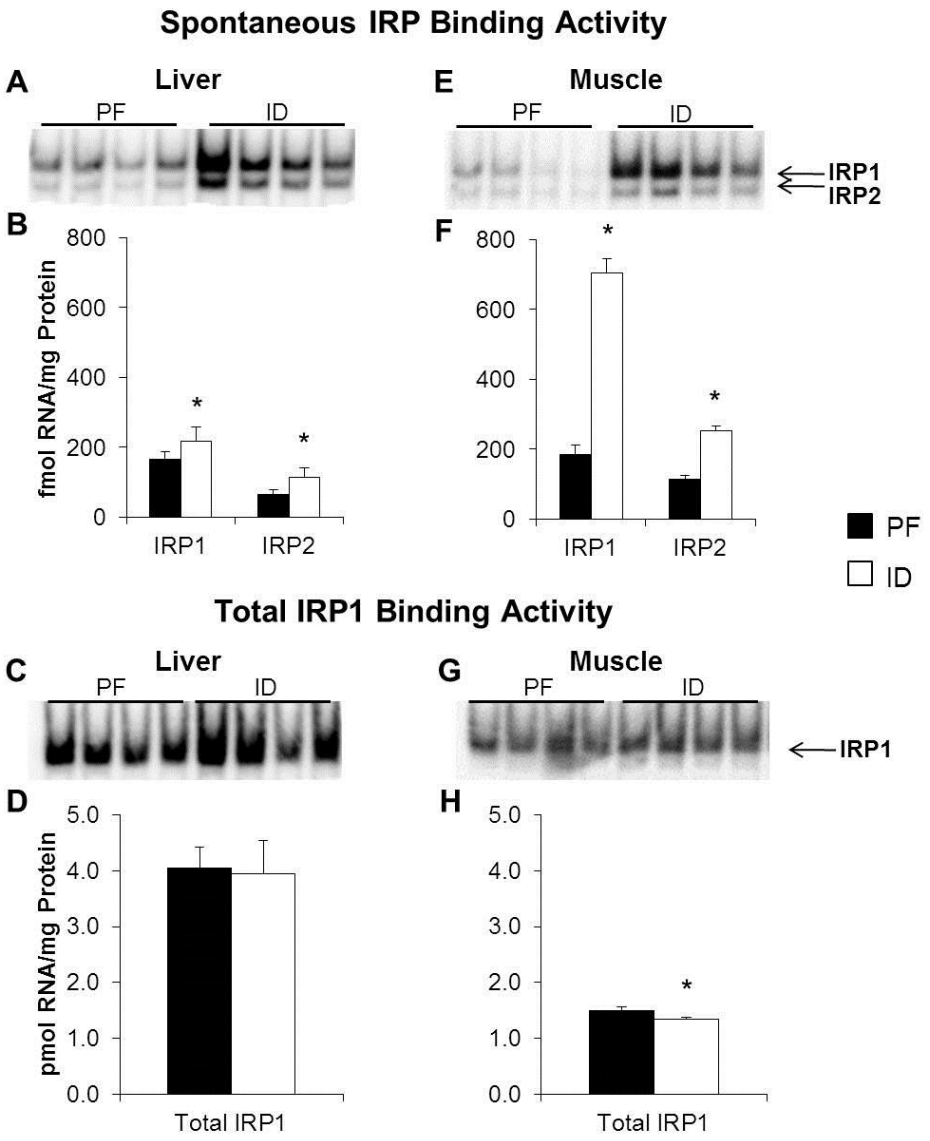

Figure 1 Spontaneous and total Iron Regulatory Protein (IRP) RNA binding activity of livers (**A** and **C**) and muscles (**E** and **G**) of pair-fed (PF) and iron-deficient (ID) rats. Quantitative analysis of spontaneous IRP1 and IRP2 RNA binding activity, and total IRP1 RNA binding activity in livers (**B** and **D**) and muscles (**F** and **H**). Values are shown as means ± SEM, n = 8/group. *Statistical significance between PF and ID groups (P < 0.05).

Two techniques were employed to identify miRNA that may be regulated in response to ID: a genome-wide miRNA microarray, and a low-density PCR miRNA array. Both techniques identified miR-210 as significantly increasing in liver in response to an ID diet (See supplemental figures S1 and S2). The significant increase in hepatic miR-210 was then verified by qRT-PCR and northern blot, which both showed a 2-fold increase in hepatic miR-210 expression (Figure 2A and S3). Soleus expression of miR-210 was also investigated by RT-PCR, and miR-210 was increased 4-fold in the soleus of the ID animals (Figure 2).

Bioinformatic examination of the mature miR-210 sequence has previously revealed that it is highly conserved among most species [20]. Figure 3A shows the species conservation of miR-210 for mice, rats, and humans. *In silico* prediction of potential miR-210 mRNA targets in mouse using the miRWalk target prediction website identified 395, 265, and 354 potential miR-210 targets in mice, rats, and humans, respectively. In an effort to narrow our potential targets list we chose to focus on mRNA known to be important in iron metabolism and homeostasis. Taking this approach, we identified the heme-containing protein cytoglobin (Cygb) as an interesting potential miR-210 target. The species conservation of the miR-210 seed site in Cygb mRNA among mice, rats, and humans is shown in figure 3B. Importantly, the validated miR-210 target Iron-Sulfur Cluster Scaffold Homolog (E. Coli) (Iscu) was also identified utilizing these methods, and was utilized as a positive control in the remaining experiments [18, 21].

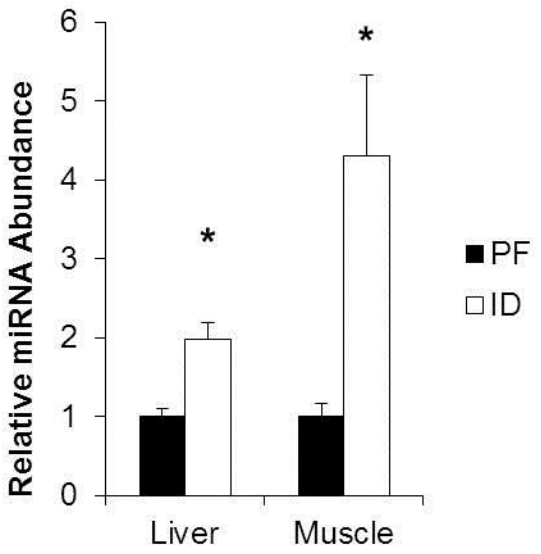

Figure 2 Assessment of miR-210 expression by qRT-PCR in livers and muscles of pair-fed (PF) and iron deficient (ID). miRNA levels were normalized to the small nuclear RNA, SNORD95, as the invariant control. Values are shown as means ± SEM, n = 8/group. *Statistical significance between PF and ID groups ($P < 0.05$).

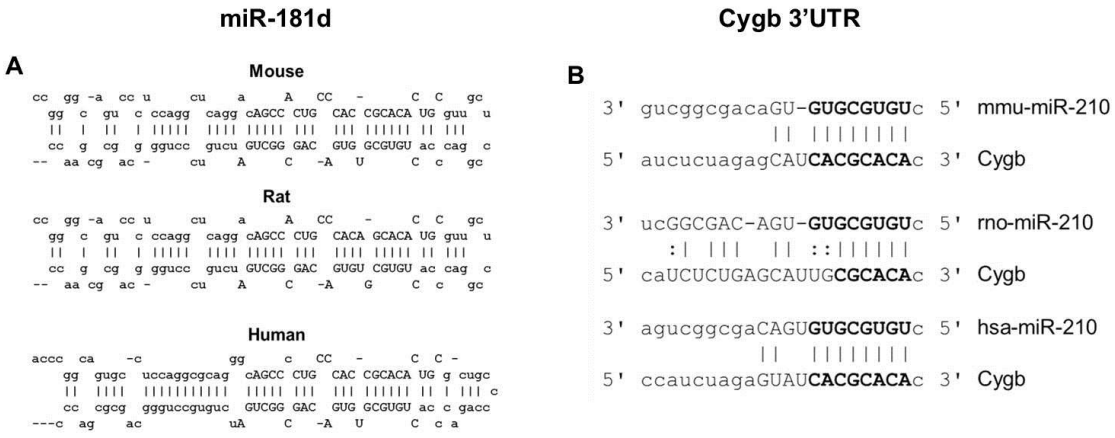

Figure 3 Species conservation of miR-210 stem-loop structure and sequence among mice, rats and humans (**A**). Species conservation of the miR-210 seed sequence in the 3'UTR of Cygb in mice, rats, and humans (**B**).

The interaction between miR-210 and Cygb was confirmed utilizing reporter assays wherein the 3'UTR of the known (Iscu) and predicted (Cygb) miR-210 targets were cloned downstream of a luciferase reporter gene, and interaction was confirmed by a significant reduction of luciferase activity. Transfection of a mature 22 nt miRNA sequence that is not predicted to regulate any known mRNA was used as a negative control (NC). Transfection of mature miR-210 (mimic) resulted in a 40% and 30% reduction in luciferase activity in the Iscu and Cygb constructs, respectively (Figures 4A and 4B). Importantly, transfection of the reporter constructs alone (control), or the reporter constructs with the NC miRNA had no effect on Iscu or Cygb luciferase activity (Figures 4A and 4B). To show target specificity, we also transfected luciferase reporters containing the 3'UTR of Iscu or Cygb with mutations in the predicted miR-210 seed sequences, (Mutant; see table S3) and saw no change in mutant luciferase activity with any treatment.

To examine the physiologic effects of up-regulated miR-210 expression in vivo, we measured the mRNA expression of Iscu and Cygb in the livers and skeletal muscles of PF and ID animals. Hepatic Iscu expression was unchanged, but Cygb expression was significantly decreased, albeit mildly, in response to dietary ID (Figure 5A). No effect on Iscu or Cygb mRNA expression was observed in skeletal muscle following 21 d on the ID diet (Figure 5B). To confirm that the livers and muscles were "sensing" iron deficiency, the mRNA abundance of iron uptake protein, transferrin receptor (Tfrc) and the iron-sensing peptide hormone, Hepcidin (Hamp1) was assessed. As expected, dietary ID resulted in a significant repression (> 99%) in hepatic hepcidin mRNA abundance and a significant increase (3.5-fold) in skeletal muscle Tfrc abundance (Figure 5A and B).

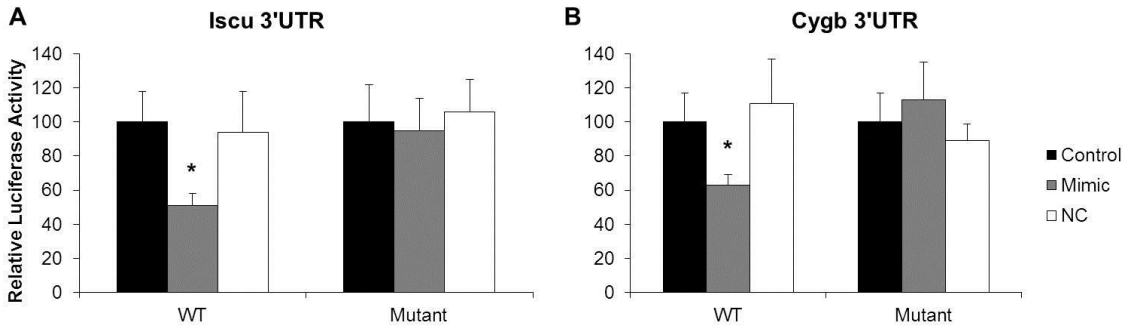

Figure 4 Validation of Iscu and Cygb as miR-210 target genes. HEK293T cells were co-transfected with the pMIR-REPORT β-Gal control vector and the pMIR-REPORT Luciferase vector containing either the wild-type (WT) or mutated (Mutant) 3'UTR of Iscu (**A**) or Cygb (**B**) (see Supplemental Table 1 for localization of predicted miR-210 binding sites and mutated sequence). Cells were then treated with vehicle (Control), a miR-210 mimetic (Mimic) or a negative control (NC) miRNA with no known predicted mRNA targets. After 24 h luciferase activity was measured and normalized to β-Galactose activity. Experiments were performed at least three times, and results are presented as fold-change ± SEM of the control transfected cells. *Statistical difference due to treatment, ($P < 0.05$).

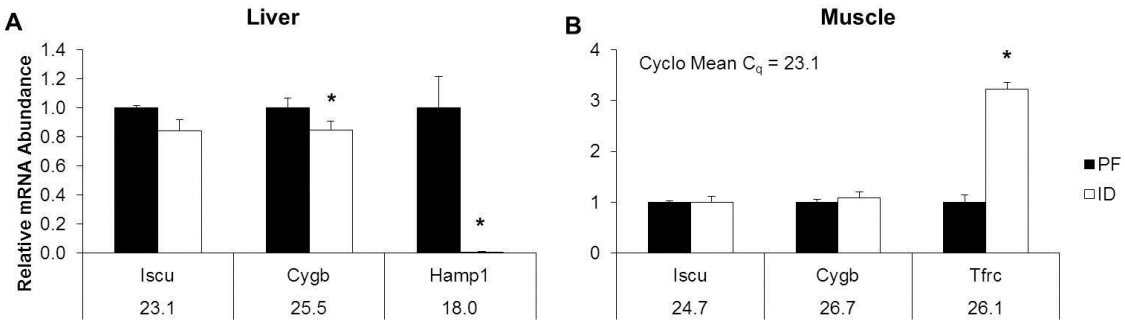

Figure 5 Expression Iscu and Cygb mRNA in the liver (**A**) and muscle (**B**) of pair-fed (PF) and iron-deficient (ID) rats. Expression of the iron-responsive hepcidin (Hamp1) and transferrin receptor (TfRc) were assessed as positive controls in liver and muscle, respectively. mRNA levels were normalized to Cyclophilin B (Cyclo) as the invariant control. Numbers beneath the gene names indicate C_q value obtained for the PF group. Values are shown as means ± SEM, n = 8/group. *Statistical significance between PF and ID groups, ($P < 0.05$).

miRNA induced alterations in target gene expression to physiologic cellular events, such as reduced nutrient availability are often times mild at best, and the timing of these responses are not fully characterized. Therefore, because the ID animals were only moderately anemic, and because tissues were only harvested at one time point, it is possible that the degree of miR-210 dependent regulation of Iscu and Cygb was too mild to be observed in our physiological model. Likewise, an adaptation to the reduced iron status may have already occurred, and as such Iscu and Cygb expression levels could have normalized. To sidestep these potential confounding factors we examined Iscu and Cygb expression in response to iron depletion in the mouse liver and muscle derived cell lines, Hepa1-6 and C2C12, respectively. Following 18 hr of treatment with the iron chelator, desferrioxamine (DFO), miR-210 expression was increased 6- and 7-fold in Hepa1-6 and C2C12 cells, respectively. The DFO induced miR-210 expression coincided with a significant repression in Iscu and Cygb mRNA abundance in C2C12 cells, and a trend toward the decreased expression of Iscu in Hepa1-6 abundance (P = 0.08) (Figure 6A and B).

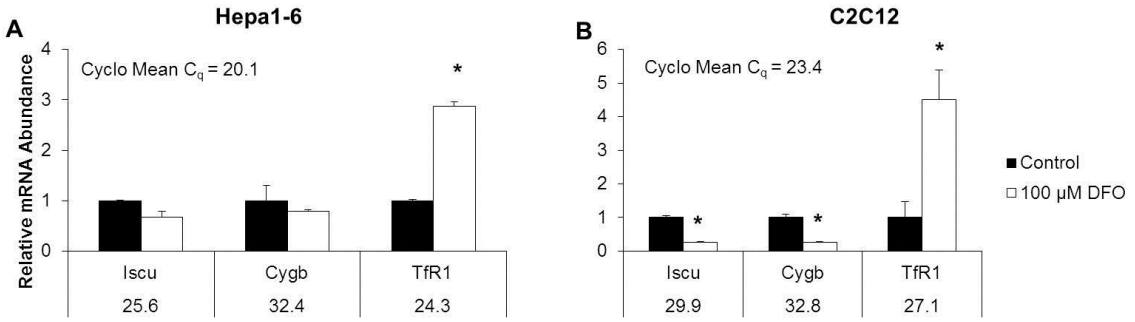

Figure 6 Expression of Iscu and Cygb mRNA in mouse hepatoma (Hepa1-6) cells (A) and mouse myoblast (C2C12) cells (B). Cells were left untreated (Control) or were treated with 100µM desferrioxamine (DFO) for 18 hr. mRNA levels were normalized to Cyclophilin B (Cyclo) as the invariant control. Numbers beneath the gene names indicate C_q value obtained for the control cells. Expression of the iron responsive transferrin receptor (Tfrc) was assessed as a positive control in both cell types. Experiments were performed at least three times, and values are shown as means ± SEM, *n* =3/group. *Statistical significance between control and DFO treatments, ($P < 0.05$).

Discussion

Anemia was confirmed in the ID animals following 21 d on a low iron diet by significant reductions in hemoglobin, hematocrit, serum iron, and liver iron. Tissue iron deficiency was further evidenced by significant increases in hepatic and skeletal muscle IRP RNA binding activity. It is well established that the effects of iron deficiency on skeletal muscle are much more pronounced than other tissues [6-8, 22]. Here too we show IRP1 binding activity in the livers of iron-deficient animals only increases to about 10% of the available pool of IRP1 protein, while in skeletal muscle, over 50% of the available IRP1 protein pool is converted to the active IRE binding form in response to ID. Also, while total IRP1 RNA binding activity was not affected by ID, skeletal muscle total RNA binding activity was significantly reduced.

Although interconversion of IRP1/c-acon via assembly and disassembly of the Fe-S cluster is thought to be the primary mechanism through which the protein's activity is regulated, IRP1 activity can also be regulated independently of iron by other means including oxidative stress and post-translational modification [23-25]. For example, when Fe-S cluster assembly is impaired, IRP1 activity is regulated through iron-dependent protein degradation [24]. Current evidence is supportive of the hypothesis that miRNA could be involved in the maintenance of iron homeostasis, as well as in the tissue specific responses to alterations in iron status [26]. Here we show that the hypoxia regulated miR-210 is also regulated in an iron-dependent manner through increased expression of the mature miR-210 in the livers and skeletal muscles of ID animals. These findings are very interesting as miR-210 has been demonstrated to be directly involved in iron utilization and regulatory pathways through direct interaction with the Fe-S cluster

assembly proteins, Iscu1/2, and because of the intricate relationship between oxygen and iron homeostasis [18, 21].

Excitingly, we have also identified and validated a novel miR-210 target, Cygb that has also been demonstrated to be intimately involved in iron utilization and hypoxic signaling pathways. It is logical then that oxygen carrying hemoprotein Cygb may be regulated by a hypoxic and iron-dependent miRNA in an effort to coordinate oxygen and iron utilization with availability [27]. For example, with insufficient iron availability, hemoglobin production falls, red blood cell formation is diminished, and oxygen carrying capacity of the blood is reduced [28]. Conversely, when oxygen availability is limited, red blood cell formation is enhanced in an effort to increase oxygen transport and tissue oxygen availability for maintenance of oxygen utilization [29, 30]. However, under iron-deficient conditions, enhanced red blood cell production would only further deplete low iron stores. It has previously been established that IRP can down-regulate hypoxic signaling, presumably in an effort to conserve diminishing iron stores [31, 32]. The iron-dependent regulation of miR-210 may then serve as a means of even further fine-tuning these regulatory processes in an effort to maintain a physiologic equilibrium between oxygen and iron usage with availability.

Based on the findings presented herein, it is tempting to postulate the observed tissue-specific effects on IRP1 RNA binding activity are the result of iron-dependent modulation of miR-210 expression and the potential downstream effects the Fe-S cluster machinery. In this study however, we did not observe a significant repression in Iscu mRNA abundance in liver or skeletal muscle. In mammals, miRNA are thought to primarily regulate gene expression via transcript degradation, however miRNA can also

act via translational repression [33]. Although not measured here, it is possible then that even though mRNA levels were unchanged, Iscu protein abundance may still have been decreased in response to dietary ID. Contesting the idea of translation repression versus transcript degradation however, were our findings that iron depletion in Hepa 1-6 and C2C12 did result in decreased Iscu mRNA abundance. Therefore, the mode of *in vivo* miR-210 mediated Iscu repression and the physiologic significance of this regulation in response to ID warrants further investigation.

The hypoxic and iron deficient induction of miR-210 and subsequent repression of Iscu likely represents an adaptive cellular response to repress mitochondrial respiration and limit iron utilization. It is somewhat counterintuitive that the iron-deficient induction of miR-210 would down-regulate Cygb expression, a well-established hypoxia induced protein [27]. Although the physiologic relevance of this response is not abundantly clear, several possibilities for this stimulus-dependent response exist. For example, it has been proposed that miR-210 is up-regulated to repress genes that are no longer necessary under hypoxic conditions, and that it remains elevated even after homeostasis has been restored to ensure maintenance of this adaptive response [20, 34]. Iron deficiency does result in decreased oxygen carrying capacity of the blood, and thus reduced oxygen delivery to tissues [35, 36]. In this study, tissues were only harvested at one time point, so it is possible that an adaptive responsive to changes in cellular oxygen tensions has already occurred, and we are observing residual miR-210 up-regulation for adaptive maintenance purposes. Once oxygen homeostasis has been restored, enhanced Cygb expression would no longer be necessary, thus a miR-210 mediated repression of Cygb would be a logical physiologic response.

Another explanation for elevated levels of miR-210 and Cygb under hypoxic conditions, but miR-210 induced Cygb repression under iron-deficient conditions could be the result of competitive inhibition of Cygb repression by other miR-210 targets in response to hypoxia. Recently, multiple studies have shown that potentially any mRNA with miRNA targets sites can function as a competing endogenous RNA (ceRNA) [33]. As mentioned earlier, miR-210 has predicted binding sites in hundreds of different genes, many of which may also be up-regulated under low oxygen conditions. Therefore, hypoxic induction of these potential miR-210 targets could effectively titrate miR-210 away from Cygb in the initial stages of hypoxia, preventing Cygb repression until hypoxic adaptation has occurred and cellular homeostasis has been restored. The newly defined long non-coding RNA (lncRNA) have been called prime candidates to serve as miRNA decoys, and may represent an as yet unidentified means of miR-210 regulation as well since most target prediction programs don't include lncRNA in their target search algorithms [33].

Ever since miR-210 was first demonstrated to target Iscu, it has been hypothesized that miR-210 could play a significant role in the regulation of Fe-S cluster containing proteins, such as IRP1 [18, 21]. While the findings of this study do not refute this hypothesis, they don't provide overwhelming evidence in support of it either. Here we show that even though miR-210 was up-regulated to a higher degree in skeletal muscle than in liver, and that total IRP1 RNA binding activity was decreased in skeletal muscle, no effect was observed on Iscu expression in our animal model. However, iron depletion in the muscle derived C2C12 cell line did result in significant increase in miR-210 expression and a corresponding repression of Iscu mRNA abundance. Thus, the

functional consequences of miR-210 mediated Iscu repression and Fe-S cluster protein regulation in response to iron deficiency remain unclear, but will likely be the subject of future investigations. We were able however to identify and validate a novel miR-210 target, Cygb, and demonstrate its functional repression in the livers of iron deficient animals, as well as in the C2C12 cell line. These findings are exciting as they extend the known functional roles of miR-210, and provide some of the first substantial evidence for the contribution of miRNA in coordinating molecular iron homeostasis in a physiologic model of dietary iron deficiency.

Materials & methods

Weanling 21 d old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing approximately 50 g were fed a purified powdered control (C) diet containing 40 mg Fe/kg for 3 d to acclimate to laboratory conditions and facilities. On d 4 (or d 0 of the experimental period), rats were randomly assigned to one of three treatment groups (n = 8/group) for the 21 d: iron deficient (ID; < 5 mg Fe/kg diet), control (C; 50 mg Fe/kg diet), or pair-fed (PF; fed the C diet at the level of intake of the ID group). The diets were purchased from Harlan Teklad (Madison, WI; C-TD.89300 and ID-TD.80396) and based on the recommendations from the American Institute of Nutrition's 1976 Standards for Nutritional Studies [37]. The inclusion of a PF group is necessary to attribute observed biological effects to a diminished iron status and not an overall decrease in overall food intake [38, 39]. For this reason, the PF group is the more appropriate control for many of the experiments described below and will be used as such.

Animal Care: All animal studies were approved by the Oklahoma State University Institutional Animal Care and Use Committee. Animals were housed at the OSU

Laboratory Animal Research Facility and maintained on a 12 h light/dark cycle in a temperature- and humidity- controlled environment in individual wire-bottomed cages to prevent coprophagy [40]. Upon completion of the treatment period, animals were anesthetized with a mixture of ketamine/xylazine (75 mg ketamine and 7.5 mg xylazine/kg body weight). Blood was collected via the abdominal aorta for plasma and serum preparation. Following cardiac exsanguinations, tissues were removed and immediately frozen in liquid nitrogen.

Assessment of Iron Status: Hemoglobin and hematocrit were assessed by Antech Diagnostics (Dallas, TX). Plasma and liver iron were determined using an ELAN 9000 ICP-Mass Spectrometer (PerkinElmer, Downers Grove, IL).

Cell culture: Mouse hepatoma (Hepa1-6) and myoblast (C2C12) cell lines (obtained from ATCC, Manassas, VA) were cultured in DMEM containing 10% heat inactivated fetal bovine serum, 5% L-glutamine, and an antibiotic-antimycotic (Cellgro, Herndon, VA) at 37ºC in 5% CO_2. Cells were treated with 100μM of the iron chelator, desferrioxamine, for 18 hr to induce iron deficiency.

IRP RNA Binding Activity: Ferritin cDNA used for IRE synthesis has been previously described by Eisenstein *et al.* [41]. Briefly, a 73 nucleotide [^{32}P]-labeled RNA containing the IRE was produced by T7 RNA polymerase, and gel purified though a 10% acrylamide 8M urea gel before determination of specific activity. Cytosolic fractions from liver and muscle were obtained by homogenizing the frozen tissues in 3 volumes HDGC buffer (20 mM HEPES, 1 mM dithiothreitol, 10 % glycerol, and 2 mM citrate) or HDGK (20 mM HEPES, 1 mM dithiothreitol, 10 % glycerol, and 175 mM potassium chloride), respectively, and collecting the supernatant after centrifugation at 100,000 x *g*.

Protein concentration was determined using the bicinchoninic acid (BCA) protein assay [42].

Spontaneous IRP1 and IRP2 RNA binding activity was assessed by incubating 5 µg cytosolic extract for 10 minutes on ice with saturating levels of [^{32}P]-labeled RNA followed by electrophoresis through a 4% polyacrylamide (60:1 acrylamide/bisacrylamide) at 150 V for ~ 75 min. Total IRP1 RNA binding activity was measured by incubating 1 µg cytosolic extract with saturating levels of [^{32}P]-labeled RNA in the presence of 6% β-mercaptoethanol at room temp for 20 min, followed by electrophoresis as described above. Gels were visualized using a Bio-Rad Phosphor K imaging screen and Personal Molecular Imager FX imaging system (Bio-Rad, Hercules, CA) for assessment of RNA binding with OptiQuant Acquisition & Analysis software (Packard Bioscience, Meridien, CT).

RNA Extraction: Total RNA was isolated from liver and skeletal muscle using RNA STAT-60 (TelTest, Inc., Friendswood, TX) following the manufacturer's protocol. RNA concentration was determined by spectrophotometric analysis at OD_{254} using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Sample purity and integrity were determined by spectrophotometric analysis ($OD_{260/280} > 1.8$ and $OD_{260/230} > 1.0$) and by agarose gel electrophoresis, respectively.

miRNA Microarray Analysis: Total RNA from the livers and soleus muscles of ID and PF animals were comparatively analyzed by LC Sciences (Houston, TX). A PF and an ID sample was compared on each miRNA array chip for a total of 8 chips each for liver and muscle analysis (n=4/group, n=8/tissue). Individual chip data was then combined, and an in-depth statistical analysis of microarray data was conducted by LC Sciences.

miRNA focused PCR array and qPCR: Alterations in liver miRNA expression were also examined using the miFinder miRNA PCR array for rat and miScript Primer Assays for individual mature RNA (SABiosciences, Valencia, CA). Briefly, 500 ng total liver and soleus RNA was reverse transcribed using the miScript II RT kit (SABiosciences), which selectively facilitates conversion of mature miRNA to cDNA to minimize background interference from longer RNA. The cDNA was then used as a template for qPCR according to the array instructions using SYBR green chemistry on an ABI 7900HT system (Applied Biosystems, Grand Island, NY). Array data was analyzed using miScript miRNA PCR Array Data Analysis Software at http://www.sabiosciences.com/mirnaArrayDataAnalysis.php. Relative quantification of each miRNA was determined by normalizing to RNU6 and 4.5S, then comparing PF and ID groups using the $2^{-\Delta\Delta Ct}$ method. Expression changes of the mature miR-210 were validated in liver and assessed in muscle, and assessed in Hepa1-6 and C2C12 cells using miScript Primer Assay with cDNA synthesized as above and analyzed using the $2^{-\Delta\Delta Ct}$ method with the small nuclear RNA, SNORD95 as the invariant control. For target mRNA relative expression determination, total liver and soleus RNA was first DNase I (Roche, Indianapolis, IN) treated and then reverse transcribed using SuperScript II (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed using an ABI 7900HT system (Applied Biosystems, Foster City, CA) and SYBR green chemistry. Relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method with Cyclophilin B (Cyclo) as the invariant control.

Bioinformatics approach for identification of potential miRNA targets: To identify potential miRNA/mRNA interactions the publically available databases miRWalk

(http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) and miRanda (www.microRNA.org), were utilized. The miRWalk program employs its own algorithm and additionally provides a comprehensive output of miRNA/mRNA interaction predictions from 8 established miRNA target prediction programs [43]. The miRanda program uses the mirSVR regression method for predicting potential miRNA/mRNA interactions, and has been described elsewhere [44]. In an effort to improve the likelihood of predicting a true target, predictions were restricted to the identification of potential targets in at least 3 of the available databases, with a minimum seed length of 7 nt and a p-value < 0.05.

Reporter Assays: Target 3'UTRs were amplified from mouse genomic DNA and cloned into the pMIR-REPORT Luciferase vector (Ambion, Austin, TX) between the SpeI/HindIII sites. Site directed mutagenesis of the miRNA seed sequences were performed by Mutagenex (Somerset, NJ). (See supplemental table S3 for predicted and mutated seed sequences). All constructs were verified by sequencing. HEK293T cells were plated at 3×10^4 cells/well and transfected 24 hours later with 50 nM miRVana miRNA mimic or scrambled control (Ambion), 100 ng of the pMIR-REPORT Luciferase vector containing the wild-type or mutant 3'UTR, and 50 ng of pMIR-REPORT β-gal Control Plasmid. Luciferase and β-galactosidase activities were assessed 24 hours after transfection using a Synergy HT microplate reader and Gen5 v 2.01 software (BioTek; Winooski, VT). Luciferase activity was normalized to β-galactosidase activity for each well.

Statistical Analysis

The significance of treatment effects was determined by ANOVA and Student's T-test techniques using SPSS software version 17.0 (IBM-SPSS, IL). All tests were done at the 95% confidence level (alpha = 0.05). Descriptive statistics were calculated on all variables to include, mean, standard deviation, and standard error of the mean. Microarray results were log-transformed and ranked by log-change between PF and ID samples. The results from each chip were combined by group and analyzed for significance using Student's T-test

References

1. Collins, J.F., M. Wessling-Resnick, and M.D. Knutson, *Hepcidin regulation of iron transport.* J Nutr, 2008. **138**(11): p. 2284-8.
2. Anderson, C.P., et al., *Mammalian iron metabolism and its control by iron regulatory proteins.* Biochim Biophys Acta, 2012. **1823**(9): p. 1468-83.
3. Salahudeen, A.A., et al., *An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis.* Science, 2009. **326**(5953): p. 722-6.
4. Rouault, T.A., et al., *Structural relationship between an iron-regulated RNA-binding protein (IRE-BP) and aconitase: functional implications.* Cell, 1991. **64**(5): p. 881-3.
5. Lill, R., *Function and biogenesis of iron-sulphur proteins.* Nature, 2009. **460**(7257): p. 831-8.
6. Ackrell, B.A., et al., *Effect of iron deficiency on succinate- and NADH-ubiquinone oxidoreductases in skeletal muscle mitochondria.* J Biol Chem, 1984. **259**(16): p. 10053-9.
7. Azevedo, J.L., Jr., et al., *Reciprocal changes of muscle oxidases and liver enzymes with recovery from iron deficiency.* Am J Physiol, 1989. **256**(3 Pt 1): p. E401-5.
8. Liew, Y.F. and N.S. Shaw, *Mitochondrial cysteine desulfurase iron-sulfur cluster S and aconitase are post-transcriptionally regulated by dietary iron in skeletal muscle of rats.* J Nutr, 2005. **135**(9): p. 2151-8.
9. Ross, K.L. and R.S. Eisenstein, *Iron deficiency decreases mitochondrial aconitase abundance and citrate concentration without affecting tricarboxylic acid cycle capacity in rat liver.* J Nutr, 2002. **132**(4): p. 643-51.
10. Chen, O.S., K.L. Schalinske, and R.S. Eisenstein, *Dietary iron intake modulates the activity of iron regulatory proteins and the abundance of ferritin and mitochondrial aconitase in rat liver.* J Nutr, 1997. **127**(2): p. 238-48.

11. Maguire, J.J., et al., *Effects of dietary iron deficiency of iron-sulfur proteins and bioenergetic functions of skeletal muscle mitochondria.* Biochim Biophys Acta, 1982. **679**(2): p. 210-20.
12. McLane, J.A., et al., *Physiological and biochemical effects of iron deficiency on rat skeletal muscle.* Am J Physiol, 1981. **241**(1): p. C47-54.
13. Barr, I., et al., *Ferric, not ferrous, heme activates RNA-binding protein DGCR8 for primary microRNA processing.* Proc Natl Acad Sci U S A, 2012. **109**(6): p. 1919-24.
14. Faller, M., et al., *Heme is involved in microRNA processing.* Nat Struct Mol Biol, 2007. **14**(1): p. 23-9.
15. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs.* Genome Res, 2009. **19**(1): p. 92-105.
16. Lewis, B.P., et al., *Prediction of mammalian microRNA targets.* Cell, 2003. **115**(7): p. 787-98.
17. Castoldi, M., et al., *The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice.* J Clin Invest, 2011. **121**(4): p. 1386-96.
18. Chan, S.Y., et al., *MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2.* Cell Metab, 2009. **10**(4): p. 273-84.
19. Chen, Z., et al., *Hypoxia-regulated microRNA-210 modulates mitochondrial function and decreases ISCU and COX10 expression.* Oncogene, 2010. **29**(30): p. 4362-8.
20. Huang, X., et al., *Hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation.* Mol Cell, 2009. **35**(6): p. 856-67.
21. Yoshioka, Y., et al., *Micromanaging Iron Homeostasis: hypoxia-inducible micro-RNA-210 suppresses iron homeostasis-related proteins.* J Biol Chem, 2012. **287**(41): p. 34110-9.
22. Dallman, P.R., *Biochemical basis for the manifestations of iron deficiency.* Annu Rev Nutr, 1986. **6**: p. 13-40.
23. Brown, N.M., et al., *Novel role of phosphorylation in Fe-S cluster stability revealed by phosphomimetic mutations at Ser-138 of iron regulatory protein 1.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15235-40.
24. Clarke, S.L., et al., *Iron-responsive degradation of iron-regulatory protein 1 does not require the Fe-S cluster.* Embo J, 2006. **25**(3): p. 544-53.
25. Pantopoulos, K., G. Weiss, and M.W. Hentze, *Nitric oxide and oxidative stress (H2O2) control mammalian iron metabolism by different pathways.* Mol Cell Biol, 1996. **16**(7): p. 3781-8.
26. Castoldi, M. and M.U. Muckenthaler, *Regulation of iron homeostasis by microRNAs.* Cell Mol Life Sci, 2012.
27. Oleksiewicz, U., et al., *Cytoglobin: biochemical, functional and clinical perspective of the newest member of the globin family.* Cell Mol Life Sci, 2011. **68**(23): p. 3869-83.
28. Borel, M.J., et al., *The impact of varying degrees of iron nutriture on several functional consequences of iron deficiency in rats.* J Nutr, 1991. **121**(5): p. 729-36.

29. Neubauer, J.A., *Invited review: Physiological and pathophysiological responses to intermittent hypoxia.* J Appl Physiol, 2001. **90**(4): p. 1593-9.
30. Robach, P., et al., *Strong iron demand during hypoxia-induced erythropoiesis is associated with down-regulation of iron-related proteins and myoglobin in human skeletal muscle.* Blood, 2007. **109**(11): p. 4724-31.
31. Davis, M.R., et al., *Hypoxia inducible factor-2 alpha is translationally repressed in response to dietary iron deficiency in Sprague-Dawley rats.* J Nutr, 2011. **141**(9): p. 1590-6.
32. Sanchez, M., et al., *Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency.* Nat Struct Mol Biol, 2007. **14**(5): p. 420-6.
33. Pasquinelli, A.E., *MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship.* Nat Rev Genet, 2012. **13**(4): p. 271-82.
34. Fasanaro, P., et al., *MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3.* J Biol Chem, 2008. **283**(23): p. 15878-83.
35. Davies, K.J., et al., *Muscle mitochondrial bioenergetics, oxygen supply, and work capacity during dietary iron deficiency and repletion.* Am J Physiol, 1982. **242**(6): p. E418-27.
36. Johnson, J.A., et al., *Muscle mitochondrial ultrastructure in exercise-trained iron-deficient rats.* J Appl Physiol, 1990. **68**(1): p. 113-8.
37. *Report of the American Institute of Nutrition ad hoc Committee on Standards for Nutritional Studies.* J Nutr, 1977. **107**(7): p. 1340-8.
38. Beard, J.L., C.S. Zhan, and D.E. Brigham, *Growth in iron-deficient rats.* Proc Soc Exp Biol Med, 1995. **209**(1): p. 65-72.
39. Davis, M.R., et al., *Enhanced expression of lipogenic genes may contribute to hyperglycemia and alterations in plasma lipids in response to dietary iron deficiency.* Genes Nutr, 2012.
40. Neale, R.J., *Coprophagy in iron-deficient rats.* Lab Anim, 1982. **16**(2): p. 204-7.
41. Eisenstein, R.S., et al., *Iron-responsive element-binding protein. Phosphorylation by protein kinase C.* J Biol Chem, 1993. **268**(36): p. 27363-70.
42. *Protein Assay Technical Handbook.* 1999, Rockford: Pierce Chemical Company.
43. Dweep, H., et al., *miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes.* J Biomed Inform, 2011. **44**(5): p. 839-47.
44. Betel, D., et al., *Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites.* Genome Biol, 2010. **11**(8): p. R90.

CHAPTER VI

MICRORNA PROFILING REVEALS MIR-181D CONTRIBUTES TO THE METABOLIC ADAPTATION TO IRON DEFICIENCY

Note: The following manuscript is a work in progress that is being considered for submission to the FASEB Journal or Physiologic Genomics

Abstract

In addition to their central role in cellular energy metabolism and ATP generation, mitochondria are essential for heme biosynthesis and biogenesis of iron-sulfur (Fe-S) clusters. Thus, mitochondrial iron demand is a key effector in the maintenance of cellular iron homeostasis. Insufficient cellular iron impairs mitochondrial function and disrupts oxidative metabolism, though the mechanisms for coordinating energy metabolism, mitochondrial iron utilization, and cellular iron availability are only beginning to be understood. Recent evidence suggests that post-transcriptional and post-translational processes are essential for mitochondrial adaptation to changes in cellular conditions. MicroRNA (miRNA) are a class of small regulatory molecules that mediate post-transcriptional gene regulation to assist in fine-tuning the response to changes in the cellular environment. Further, miRNA may also function as nutrient sensors and regulators of mitochondrial activity, and thus are likely candidates for coordinating mitochondrial function with iron availability. In this study, we examined changes in miRNA expression in response to iron deficiency and found the mitochondrial associated miRNA, miR-181d is significantly elevated in the livers and skeletal muscles of iron deficient animals. Further bioinformatic analyses and *in vitro* reporter assays confirmed that miR-181d contributes to the regulation of two mitochondrial proteins important for iron trafficking and cellular energy metabolism, the mitochondrial iron transporter mitoferrin 1 (Slc25a37) and the rate-limiting enzyme of β-oxidation in the muscle mitochondria, carnitine palmitoyltransferase 1B (Cpt1b). These findings reveal that expression of miR-181d is subject to regulation by iron status and that this miRNA may contribute to altered fuel utilization associated with iron deficiency.

Introduction

As a result of their central roles in Fe-S cluster biogenesis and heme production, mitochondria are a major site for cellular iron utilization [1, 2]. Indeed, iron is required for mitochondrial energy production through both heme- (cytochromes) and Fe-S cluster-containing proteins (aconitase). Thus, it is important that cellular iron homeostasis and mitochondrial iron availability be tightly coordinated to preserve optimal mitochondrial function. Recently, the central regulators of cellular iron homeostasis, iron regulatory proteins (IRP) 1 and 2, were identified as playing an essential role in mitochondrial iron availability as their presence in the cytosol is necessary to protect the mitochondria from detrimental iron deficiency [3]. However, the signaling mechanisms coupling cytosolic and mitochondrial iron homeostasis are not fully clear, except to suggest that cytosolic iron metabolism is strongly influenced by mitochondrial iron demand [1, 2].

Iron deficiency progresses in stages beginning with the depletion of iron stores, followed by diminished erythropoiesis, and finally a reduction in hemoglobin production, the hallmark of iron deficiency anemia [4]. Symptoms of iron deficiency include weakness, fatigue, and a reduced capacity to transport oxygen as a result of lowered hemoglobin levels [5]. Indeed, many of the negative effects associated with iron deficiency arise from the reduced oxygen carrying capacity of the blood as well as the compromised respiratory capacity of the skeletal muscle as a result of insufficient iron for TCA cycle and electron transport flux [6]. Despite compensatory mechanisms employed by the liver, such as increasing non-iron-containing enzyme activities, a metabolic shift occurs in which the body becomes decreasingly reliant on β-oxidation for energy production, and subsequently more dependent on glucose utilization and gluconeogenesis

[7-10]. The decrease in muscle oxidation activity is the result of decreased mitochondrial enzyme content, rather than decreased enzyme activity, suggestive of insufficient mitochondrial iron availability to support heme biosynthesis and production of iron-sulfur (Fe-S) clusters for Fe-S containing proteins located in either the mitochondria or the cytoplasm [6, 11].

Mitochondrial activity and function, and thus iron needs, are largely influenced by changes in the cellular environment. When cellular iron status is impaired and iron availability is limiting, both mitochondrial protein levels and oxidative capacity decrease [6, 11]. In addition to these changes in mitochondrial enzyme function, mitochondrial biogenesis is also impaired in response to iron deficiency [6, 11, 12]. Current evidence suggests that post-transcriptional and post-translational processes are critical for mitochondrial adaptation to iron limiting conditions [12, 13]. A potentially important post-transcriptional mechanism contributing to this mitochondrial adaptation involves microRNA (miRNA). MiRNA are a class of small (~22-nt) non-coding RNA that function to fine-tune gene expression through mechanisms involving enhanced mRNA decay and/or translational repression [14-16]. Because miRNA expression may be affected by nutrient availability, miRNA-dependent control of iron metabolism is an attractive model to explain some of the observed changes in mitochondrial function associated with iron deficiency [17, 18].

Furthermore, *in vitro* studies have demonstrated that the oxygen sensitive miRNA-210 can regulate cellular metabolism via direct inhibition of the Fe-S cluster assembly proteins (ISCU1/2) [19, 20]. Also, inhibition of the liver-specific miRNA-122 has established its importance in both lipid and iron homeostasis in mice, and provided

additional evidence for the role of miRNA to function as important regulators in the response to alterations in nutrient homeostasis [21, 22]. To date however, a physiologically-based study investigating the potential for dietary iron deficiency to influence miRNA expression and regulation has not been conducted. Furthermore the roles of miRNA in the regulation of iron homeostasis and the adaptive response to deficiency remain unknown. Thus our primary objectives were to determine the extent to which expression of miRNA is regulated in response to dietary iron deficiency and to characterize the impact of miRNA expression on potential regulatory targets involved in iron homeostasis and energy metabolism. To do this we first examined the miRNA profiles of iron-adequate and iron-restricted conditions in animals. We then focused on miR-181d, a miRNA with direct implications in the iron-deficient response, and were able to identify and validate two novel miR-181 targets critical for the metabolic adaptation to iron deficiency.

Methods

Weanling 21 d old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing approximately 50 g were fed a semi-purified powdered control diet containing 40 mg Fe/kg for 3 d to acclimate to laboratory conditions and facilities. On d 4 (or d 0 of the experimental period), rats were randomly assigned to one of two treatment groups (n = 8/group) for 21 d: iron deficient (ID; < 5 mg Fe/kg diet) or pair-fed (PF; fed the control diet at the level of intake of the ID group). The diets were purchased from Harlan Teklad (Madison, WI; C-TD.89300 and ID-TD.80396) and based on the recommendations from the American Institute of Nutrition's 1976 Standards for Nutritional Studies [23]. The use of a PF control group is necessary because rats fed an

ID diet may consume as much as 15% less then animals fed a control diet *ad libitum* [24, 25]. Use of a PF control then allows us to attribute observed biological effects to a diminished iron status and not an overall decrease in overall food intake.

Animal Care: All animal protocols were approved by the Oklahoma State University Institutional Animal Care and Use Committee. Animals were housed at the OSU Laboratory Animal Research Facility and maintained on a 12 h light/dark cycle in a temperature- and humidity-controlled environment in individual wire-bottomed cages. Upon completion of the treatment period, animals were anesthetized with a mixture of ketamine/xylazine (75 mg ketamine and 7.5 mg xylazine/kg body weight). Blood was collected via the abdominal aorta for serum preparation. Following cardiac exsanguination, the liver and soleus were removed and snap-frozen in liquid nitrogen.

Assessment of Iron Status: Hemoglobin and hematocrit were determined by Antech Diagnostics (Dallas, TX). Non-heme liver iron concentration was determined by colorimetric assay as previously described [26].

RNA Extraction: Total RNA was isolated from liver and soleus (skeletal muscle) using RNA STAT-60 (TelTest, Inc., Friendswood, TX) following the manufacturer's protocol. RNA concentration was determined by spectrophotometric analysis at OD_{260} using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For each sample RNA purity and integrity were determined in-house by spectrophotometric analysis at $OD_{260/280}$, and by agarose gel electrophoresis, respectively. Samples that met pre-determined quality standards ($OD_{260/280} > 1.8$ and $OD_{260/230} > 1.0$) were selected for sequencing, and RNA concentration and integrity were further assessed by Illumina, Inc. (Sand Diego, CA) using an Agilent Bioanalyzer (Palo Alto, CA).

Library Construction: Library construction was performed by Illumina, Inc. (San Diego, CA) using the Small RNA Sample Preparation Alternative version 1.5 Protocol (Part # 15002615 Rev. A). First, total RNA (N=8; n=4 from the ID group and n=4 from the PF group) isolated from liver as described above was ligated to the v1.5 small RNA 3' and 5' adapters. Next, the ligated fragments were purified by polyacrylamide gel electrophoresis and selected based on size before recovery by column centrifugation. The purified ligation products were then placed in an 8-channel flow cell lined with oligonucleotides that bind to the ligated adaptor, reverse-transcribed, and PCR-amplified to specifically enrich for the fragments containing both 5' and 3' adaptors. Finally, size, purity, and concentration of generated libraries were assessed to evaluate quality before further sequencing analysis using an Agilent Technologies 2100 Bioanalyzer (Santa Clara, CA).

miRNA Sequencing: Small RNA libraries were sequenced using a Genome Analyzer IIx (Illumina, Inc., San Diego, CA). Raw sequencing reads were generated in FASTA format to facilitate downstream analysis and application.

Bioinformatics for identification of differentially expressed miRNA: In an effort to obtain unbiased and accurate results for identification of both differentially expressed and novel miRNA, raw sequencing data was analyzed independently using three previously validated bioinformatics approaches [27-29]. The software packages miRExpress and Pipeline (Illumina, Inc., San Diego, CA) were used to align sequencing reads against the rat genome and identify the differentially expressed sequences based on annotated miRNA sequences. The third technique employed a combination of alignment and

structural analytic bioinformatic techniques to generate a miRNA expression profile for each biological sample.

The computational analysis using miRExpress was performed using the software package miRExpress v 17 (http://mirexpress.mbc.nctu.edu.tw/) and the OSU High Performance Computing Center. miRExpress uses the raw sequencing data provided from sequencing output to develop a miRNA expression profile [29]. First, identical reads were merged into a unique read, and then each unique read is counted. Next, unique reads were trimmed based on the presence and location of a full or partial adaptor sequence. In the third step, sequences were aligned to the sequences of known mature miRNA using the sequencing data available from miRBase (Release 18.0), a database of all currently published and annotated miRNA sequences. Lastly, miRNA expression profiles were created by computing the sum of read counts for each miRNA according to the matched alignments from the third step [29].

The data were also analyzed for read quality and miRNA expression profiles by using Illumina Pipeline software. This software performs the alignment process using the Efficient Large-Scale Alignment of Nucleotide Databases, followed by a post-sequencing analysis from these aligned reads using the Consensus Assessment of Sequence and Variation (CASAVA) Software package. CASAVA was then used to generate descriptive statistics such as percentage chromosome coverage, and read counts for exons, genes, and splice junctions. The last step in this approach was the utilization of the software tool, Flicker v 3.0, which employs a nearly identical four step process to that described above for miRExpress to generate a miRNA expression profile.

The third computational analysis utilized has been previously described [27]. First, all miRNA reads without perfect matches to the 5' end of the adaptor sequences were removed. Then reads were aligned using Repbase (version 14, obtained from http://www.girinst.org), and known noncoding RNAs (rRNAs, tRNAs, snRNAs, soRNAS, etc.) identified by Rfam (http://www.sanger.ac.uk/Software/Rfam/ftp.shtml) with National Center for Biotechnology Information BLASTn were removed [30]. Following removal, unique sequences between 18 and 28 nt were mapped using BLASTn searches versus the rat genome and aligned to known miRNA sequences using miRBase (version 18; http://microrna.sanger.ac.uk/) to identify conserved miRNA homologs.

Bioinformatics approach for identification of potential miRNA targets: To identify potential miRNA/mRNA interactions the publicly-available database miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) was utilized [31]. The miRWalk program employs its own algorithm and additionally provides a comprehensive output of miRNA/mRNA interaction predictions from 8 established miRNA target prediction programs. In an effort to improve the likelihood of predicting a bona fide target, predictions were restricted to the identification of potential targets in at least 3 of the available databases, with a minimum seed length of 7 nt and a p-value < 0.05.

Quantitative Real-Time PCR (qPCR): Iron-dependent alterations in miR-181d expression in liver and muscle were assessed using qPCR using miScript Primer Assays (SABiosciences, Valencia, CA). Briefly, 500 ng total RNA from liver and soleus was reverse-transcribed using the miScript II RT kit (SABiosciences), which selectively facilitates conversion of mature miRNA to cDNA to minimize background interference from longer RNA. The cDNA was then used as a template for qPCR according to the

manufacturer's instructions using SYBR green chemistry on an ABI 7900HT system (Applied Biosystems-Life Technologies, Grand Island, NY). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with the snRNA, SNORD95 as the invariant control. For target mRNA relative expression determination, total liver and soleus RNA was first treated with DNase I (Roche, Indianapolis, IN) and then reverse-transcribed using SuperScript II (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using an ABI 7900HT system (Applied Biosystems, Foster City, CA) and SYBR green chemistry. Relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method with Cyclophilin B (Cyclo, also known as peptidyl isomerase B or *Ppib*) as the invariant control.

Reporter Assays: Target 3'UTRs were amplified from mouse genomic DNA and cloned into the pMIR-REPORT Luciferase vector (Ambion, Austin, TX) between the SpeI/HindIII sites. Site-directed mutagenesis of the miRNA seed sequences in 3'UTRs were performed by Mutagenex (Somerset, NJ). (See supplemental table S1 for wild-type and mutagenized seed sequences). All constructs were verified by sequencing. HEK293T (ATCC, Manassas, VA) cells were plated at 3×10^4 cells/well in a flat-bottom 96-well plate and transfected after 24 hr with 50 nM miRVana miRNA mimic or scrambled control (Ambion), 100 ng of the pMIR-REPORT Luciferase vector containing the wild-type or mutant 3'UTR, and 50 ng of pMIR-REPORT β-gal Control Plasmid. Luciferase and β-galactosidase activities were assessed 24 hr after transfection using a Synergy HT microplate reader and Gen5 v 2.01 software (BioTek; Winooski, VT). Luciferase activity was normalized to β-galactosidase activity for each well.

Statistical Analysis

The significance of treatment effects were assessed by Student's T-test techniques using SPSS software version 17.0 (IBM-SPSS, Chicago IL). All tests were performed at the 95% confidence level (alpha = 0.05). Descriptive statistics were calculated on all variables to include, mean, standard deviation, and standard error of the mean. Sequencing data were analyzed by obtaining read frequencies from PF and ID samples, and comparing them by Mann-Whitney *U*. The results from each chip were combined by group and analyzed for significance using Student's T-test.

Results

As expected, ID animals exhibited a ~ 40% reduction in both hemoglobin and hematocrit, and a 57% reduction in non-heme liver iron after 21 d on the low iron diet compared to their PF controls (Figure 1). Importantly, and in agreement with our hypothesis, this reduction in iron status resulted in significant alterations in hepatic miRNA expression (Figure 2). Interestingly, the three different bioinformatic analyses that were performed resulted in unique miRNA expression profiles between the PF and ID groups. In an effort to ensure accuracy, we chose to further examine miRNA that were identified as being differentially expressed by all three approaches (Figure 2). Additionally, only differentially-expressed miRNA that were conserved among mice, rats, and humans, were chosen as potential candidates for validation and target prediction.

One miRNA candidate meeting the criteria described above was miR-181d. Differential expression of hepatic miR-181d was then confirmed by qRT-PCR, and was also examined in the muscle (Figure 3). Relative expression of miR-181d increased ~ 4-

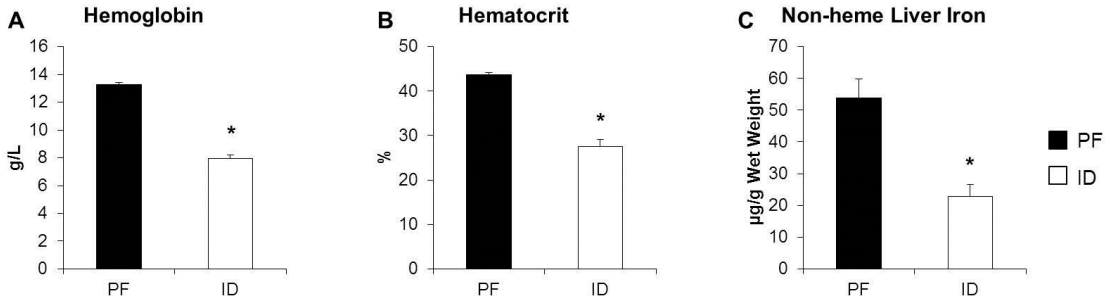

Figure 1 Hematologic indices of iron status. Hemoglobin (**A**), hematocrit (**B**), and non-heme liver iron (**C**) concentrations in pair-fed (PF) and iron deficient (ID) rats. Vales are means ± SEM, n = 8/group. *Statistical significance between PF and ID groups, (P = 0.05).

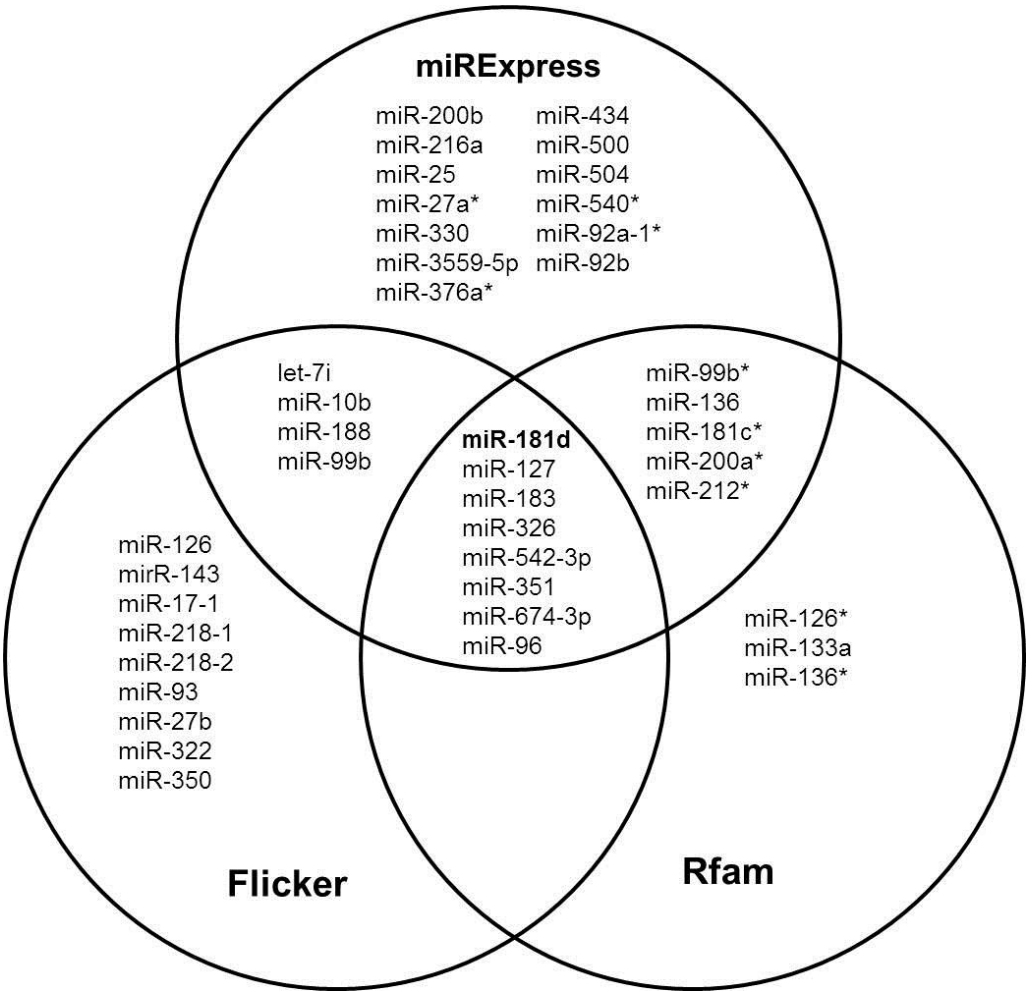

Figure 2 Venn diagram of miRNA identified as being differentially regulated in response to dietary iron deficiency by three independent bioinformatic analyses. miR-181d is highlighted because of it was found to be expressed in both liver and muscle and was predicted to target mRNA related to the iron deficient response ($P < 0.05$).

fold in the livers and ~ 2.5 fold in the soleus muscles of rats consuming the ID diet compared to the PF group (Figure 3). Further bioinformatics analyses revealed that miR-181d potentially targeted the mitochondrial iron transporter Slc25a37 (mitoferrin1) as well as the rate controlling enzyme of β-oxidation in the muscle mitochondria, carnitine palmitoyltransferase 1B (Cpt1b) (Figure 4A). Importantly, the target seed recognition site in the 3'UTR of Slc25a37 and Cpt1b was also conserved among mice, rats, and humans (Figure 4B and C).

To validate the *in silico* predictions of interactions between miR-181d and the 3'UTR of Slc25a37 and Cpt1b a reporter system was utilized. The entire 3'UTR of Slc25a37 and Cpt1b were cloned into the 3'UTR of the luciferase reporter gene in pMIR-REPORT and then co-transfected with either a mature miR-181d mimic or a negative control (NC) miRNA that is not predicted to regulate any known mRNA. Interaction was confirmed by a significant reduction in luciferase activity. Co-transfection with the miR-181d mimic reduced luciferase activity of the wild type (WT) Slc25a37 and Cpt1b reporters by ~50% and ~40%, respectively (Figure 5A and B). Transfection of the reporter construct alone or co-transfection of the reporter construct with the NC miRNA had no effect on Slc25a37 or Cpt1b luciferase activity (Figures 5A and B). To demonstrate target specificity of miRNA-mRNA interactions, luciferase activity was examined in reporters containing the 3'UTR of Slc25a37 or Cpt1b with mutations in the predicted miR-181d seed sequences (Mutant; see table S1). In the presence of a miR-181d mimic, Slc25a37 and Cpt1b mutants failed to exhibit a repression in luciferase activity.

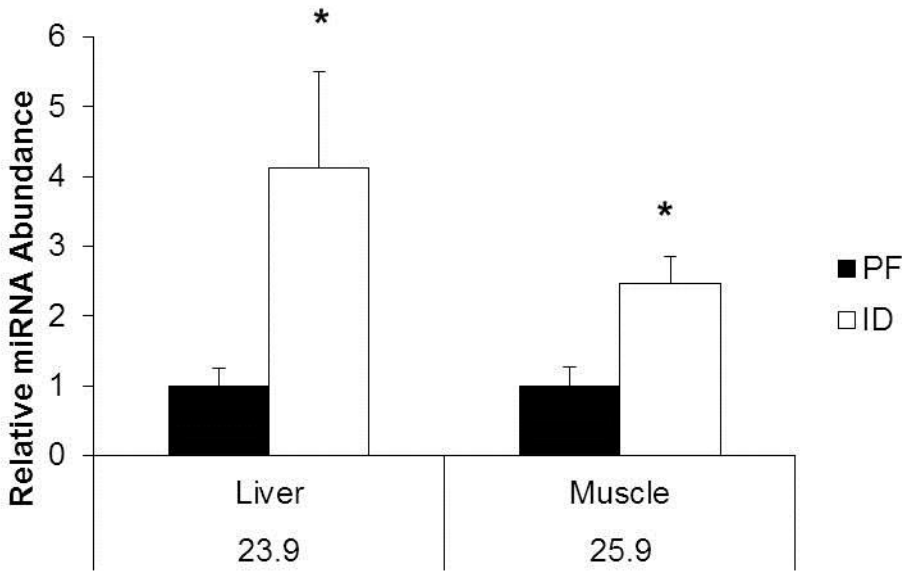

Figure 3 Validation of differential miR-181d expression in livers and muscles of pair-fed (PF) and iron deficient (ID) rats by qRT-PCR. miRNA levels were normalized to the small nuclear RNA, SNORD95, as the invariant control. Numbers beneath the gene names indicate C_q value obtained for the PF group. Values are shown as means ± SEM, n = 8/group. *Statistical significance between PF and ID groups ($P < 0.05$).

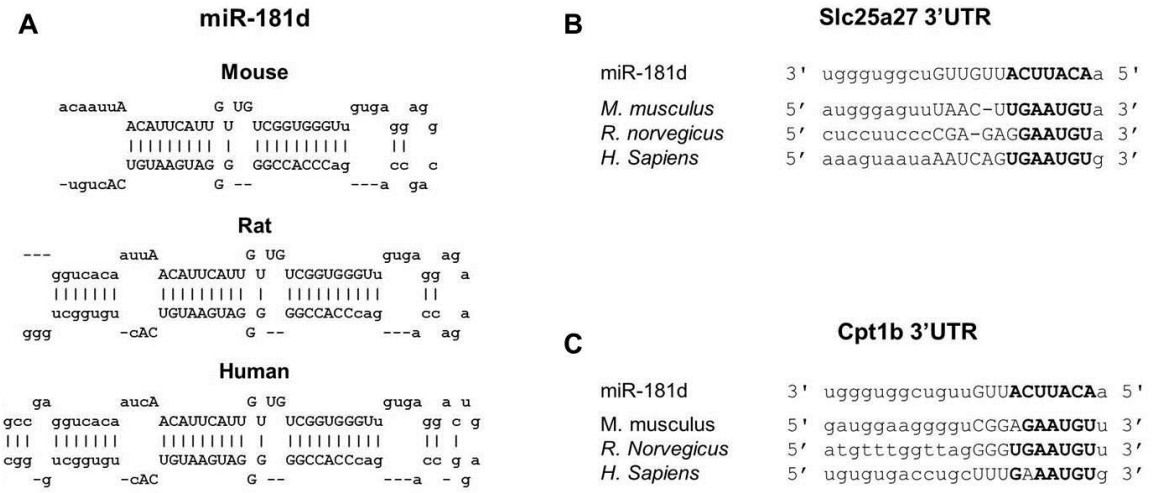

Figure 4 Species conservation of miR-181d stem-loop structure and sequence among mice, rats and humans (**A**). Species conservation of the miR-181d seed sequence in the 3'UTR of Slc25a37 (**B**) and Cpt1b (**C**) in mice, rats, and humans.

The expression of Slc25a37 and Cpt1b mRNA was then assessed in liver and soleus tissue from ID and PF animals. Although modest, the expression of Slc25a37 was significantly reduced in both tissues in ID animals compared to the PF control group (Figure 6A). Cpt1b expression was also significantly decreased in the soleus, but not the liver, of ID animals compared to the PF animals (Figure 6B). Finally, expression of the iron-responsive transferrin receptor (Tfrc) mRNA was also examined for use as a positive control because Tfrc mRNA is stabilized in response to iron deficiency resulting in increased levels of transcript [9]. Tfrc mRNA expression was increased 7- and 3.5-fold in the liver and muscle, respectively, in response to dietary ID.

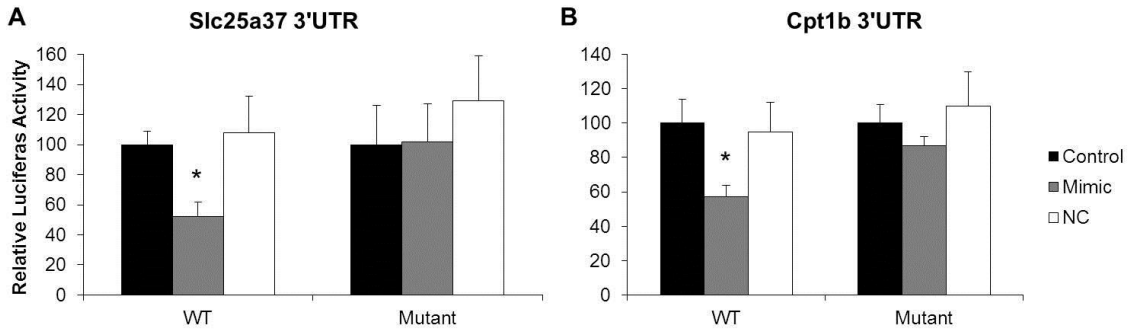

Figure 5 Validation of Slc25a37 and Cpt1b as miR-181d target genes. HEK293T cells were co-transfected with the pMIR-REPORT β-Gal control vector and the pMIR-REPORT Luciferase vector containing either the wild-type (WT) or mutated (Mutant) 3'UTR of Slc25a37 (**A**) or Cpt1b (**B**) (see Supplemental Table 1 for localization of predicted miR-210 binding sites and mutated sequence). Cells were then treated with vehicle (Control), a miR-181d mimetic (Mimic) or a negative control (NC) miRNA with no known predicted mRNA targets. After 24 h luciferase activity was measured and normalized to β-Galactose activity. Experiments were performed at least three times, and results are presented as fold-change ± SEM of the control transfected cells. *Statistical difference due to treatment, ($P < 0.05$).

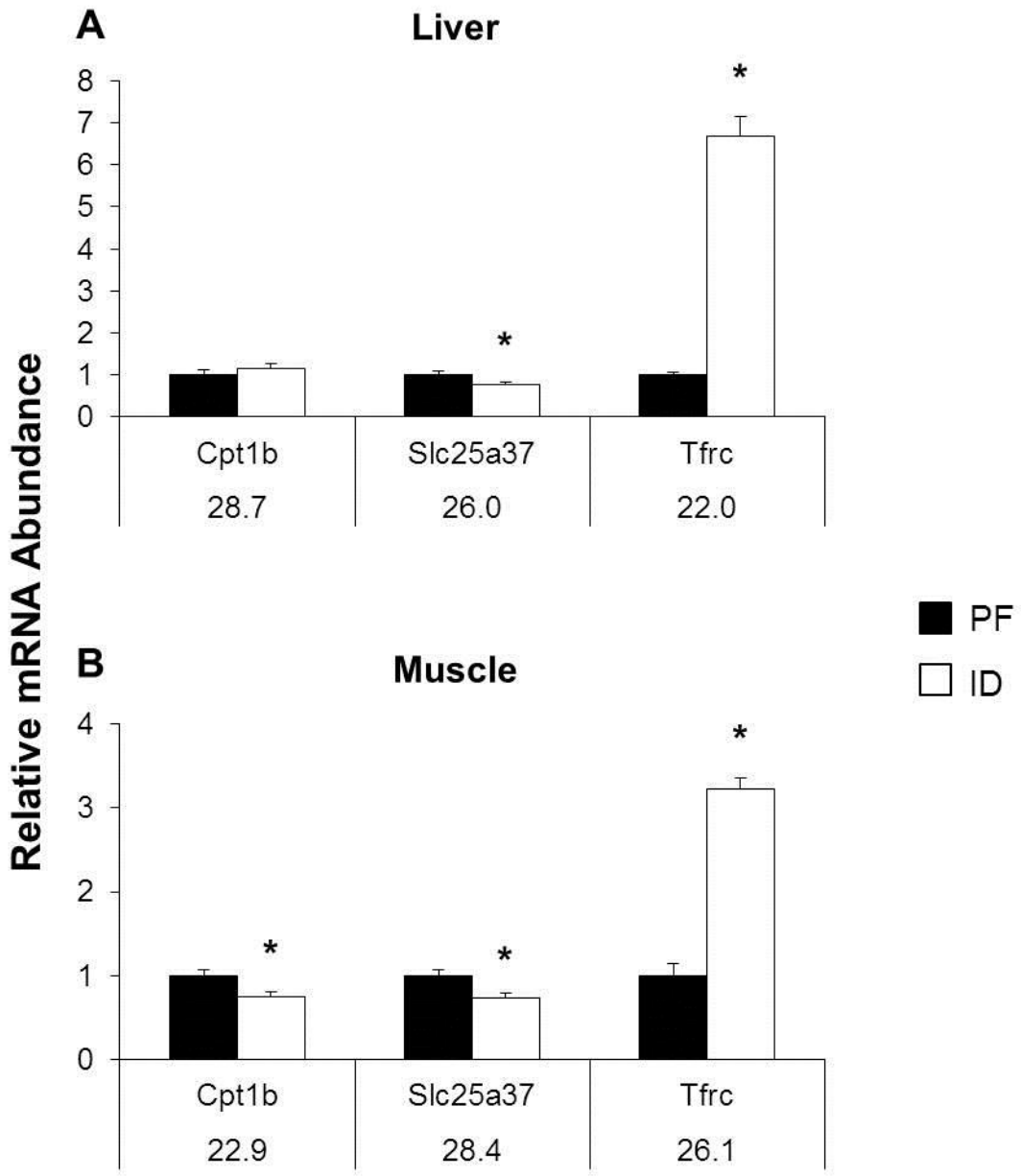

Figure 6 Expression Slc25a37 and Cpt1b mRNA in the livers (**A**) and muscles (**B**) of pair-fed (PF) and iron-deficient (ID) rats. Expression of the iron-responsive transferrin receptor (TfRc) was assessed as a positive control. mRNA levels were normalized to Cyclophilin B (Cyclo) as the invariant control. Numbers beneath the gene names indicate C_q value obtained for the PF group. Values are shown as means ± SEM, n = 8/group. *Statistical significance between PF and ID groups, ($P < 0.05$).

Discussion

Three weeks on a low iron diet was sufficient to significantly reduce hemoglobin, hematocrit, and non-heme liver iron in the ID animals. Although not measured in this study, a hemoglobin content of 80 g/L or less corresponds with significantly reduced muscle iron [32]. Previous studies have demonstrated that this level of iron deficiency is also sufficient to elicit significant metabolic effects, such as hyperglycemia and hyperlipidemia [25, 33]. Further, the severity of metabolic consequences associated with iron deficiency appears to be a graded response that progresses with decreasing hemoglobin levels as evidenced by an inverse correlation between hemoglobin levels and serum levels of lipids and glucose [34]. Changes in the abundance of mRNA involved in glucose and lipid metabolism in yeast and animals in response to iron deficiency suggest that the metabolic adaption to iron deficiency may be regulated at the level of mRNA expression and stability, but the contributing regulatory factors have yet to be fully elucidated [12, 13, 25].

miRNA have been termed "biological rheostats" of cellular function and response as a result of their capacity to elicit modest, yet biologically significant, modifications in gene expression in response to changes in environmental cues such as developmental timing events and alterations in nutrient status [35-37]. In this study, the expression of miR-181d as a miRNA was significantly elevated in the liver and skeletal muscle as a result of iron deficiency. Interestingly, the miR-181 family of miRNA was previously identified as being a modifier of mitochondrial function, and miR-181d in particular has been identified as being present in the mitochondria in mouse liver [17, 38]. In the present study, we examined the regulation of two previously uncharacterized miR-181d

mitochondrial-related targets, SLC25a37 and Cpt1b, in the liver and soleus of ID and PF animals. These two tissues were selected for analysis because they are known to exhibit tissue-dependent responses to ID, and it was of interest to assess whether miRNA were integral in this tissue-dependent regulation.

The mitochondrial iron transporter, Slc25a37 is localized to the inner mitochondrial membrane and functions as an essential mitochondrial iron importer to support mitochondrial iron demands for heme synthesis and Fe-S cluster biogenesis [2, 39]. Although Slc25a37 is most highly expressed in erythroid cells, it is expressed at detectable levels in other tissues, including both liver and skeletal muscle [2, 39]. The miR-181d directed down-regulation of Slc25a37 may represent a means of coordinating cytosolic iron availability with mitochondrial iron utilization, and therefore could play a critical role in coordinating cellular iron homeostasis in response to iron deficiency.

Questions remain however as to whether alterations in fuel utilization observed in ID animals are the result of a physiologic adaptation to iron deficiency, or pathologic consequences of insufficient iron availability. The reduction in Cpt1b mRNA observed in the skeletal muscle of ID animals is consistent with a role of miR-181d in controlling Cpt1b expression. In fact, the miR-181d-dependent regulation of Cpt1b may explain the significant reduction in skeletal muscle Cpt1b enzyme activity and diminished β-oxidation previously observed in response to ID [40]. Additionally, miR-181d targeted down-regulation of Cpt1b could also contribute to the intramuscular lipid droplet accumulation observed in rodent models of iron deficiency [41]. Although the consequence of lipid accumulation in the skeletal muscle of ID animals has not been directly assessed, skeletal muscle lipid deposition in humans is associated with negative

metabolic effects, including the development of skeletal muscle insulin resistance [42, 43]. Therefore, the therapeutic targeting and inhibiting of miR-181d to enhance Cpt1b activity is an intriguing possibility for the treatment of metabolic disorders associated with lipid accumulation and insulin resistance [44].

Iron deficiency results in a multitude of deleterious physiological consequences, many of which can be directly linked to the severe negative impacts it has on skeletal muscle. Indeed, skeletal muscle is one of the tissues of the body that appears to be particularly sensitive to iron deficiency. As iron deficiency progresses in severity, many of the associated pathologic consequences slowly manifest in a graded response as iron stores are depleted and hemoglobin production is severely repressed. The increased expression of miR-181d in response to iron deficiency in both liver and skeletal muscle could provide an additional mechanism that contributes to the metabolic adaption to iron deficiency. The physiologic importance of miR-181d down-regulation of Cpt1b in response to iron deficiency is of significant interest as iron deficiency is the number one micronutrient deficiency worldwide, and the potential for iron deficiency to contribute to or to exacerbate metabolic disorders is unknown. These findings have provided novel insight into metabolic adaptation to the iron deficiency and have demonstrated how miRNA contribute to the molecular coordination of iron homeostasis in a physiologic model of dietary iron deficiency.

References

1. Hentze, M.W., et al., *Two to tango: regulation of Mammalian iron metabolism.* Cell, 2010. **142**(1): p. 24-38.
2. Richardson, D.R., et al., *Mitochondrial iron trafficking and the integration of iron metabolism between the mitochondrion and cytosol.* Proc Natl Acad Sci U S A, 2010. **107**(24): p. 10775-82.
3. Galy, B., et al., *Iron regulatory proteins secure mitochondrial iron sufficiency and function.* Cell Metab, 2010. **12**(2): p. 194-201.
4. Weaver, C.M. and S. Rajaram, *Exercise and iron status.* J Nutr, 1992. **122**(3 Suppl): p. 782-7.
5. Beard, J.L., *Iron biology in immune function, muscle metabolism and neuronal functioning.* J Nutr, 2001. **131**(2S-2): p. 568S-579S; discussion 580S.
6. Dallman, P.R., *Biochemical basis for the manifestations of iron deficiency.* Annu Rev Nutr, 1986. **6**: p. 13-40.
7. Azevedo, J.L., Jr., et al., *Reciprocal changes of muscle oxidases and liver enzymes with recovery from iron deficiency.* Am J Physiol, 1989. **256**(3 Pt 1): p. E401-5.
8. Henderson, S.A., P.R. Dallman, and G.A. Brooks, *Glucose turnover and oxidation are increased in the iron-deficient anemic rat.* Am J Physiol, 1986. **250**(4 Pt 1): p. E414-21.
9. Hentze, M.W., M.U. Muckenthaler, and N.C. Andrews, *Balancing acts: molecular control of mammalian iron metabolism.* Cell, 2004. **117**(3): p. 285-97.
10. Linderman, J.K., et al., *Maintenance of euglycemia is impaired in gluconeogenesis-inhibited iron-deficient rats at rest and during exercise.* J Nutr, 1994. **124**(11): p. 2131-8.
11. Ackrell, B.A., et al., *Effect of iron deficiency on succinate- and NADH-ubiquinone oxidoreductases in skeletal muscle mitochondria.* J Biol Chem, 1984. **259**(16): p. 10053-9.
12. Rensvold, J.W., et al., *Complementary RNA and protein profiling identifies iron as a key regulator of mitochondrial biogenesis.* Cell Rep, 2013. **3**(1): p. 237-45.
13. Puig, S., S.V. Vergara, and D.J. Thiele, *Cooperation of two mRNA-binding proteins drives metabolic adaptation to iron deficiency.* Cell Metab, 2008. **7**(6): p. 555-64.
14. Alvarez-Garcia, I. and E.A. Miska, *MicroRNA functions in animal development and human disease.* Development, 2005. **132**(21): p. 4653-62.
15. Parasramka, M.A., et al., *MicroRNAs, diet, and cancer: new mechanistic insights on the epigenetic actions of phytochemicals.* Mol Carcinog, 2012. **51**(3): p. 213-30.
16. Pasquinelli, A.E., *MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship.* Nat Rev Genet, 2012. **13**(4): p. 271-82.
17. Li, P., et al., *Control of mitochondrial activity by miRNAs.* J Cell Biochem, 2012. **113**(4): p. 1104-10.
18. Ryu, M.S., et al., *Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis.* Proc Natl Acad Sci U S A, 2011. **108**(52): p. 20970-5.

19. Chan, S.Y., et al., *MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2.* Cell Metab, 2009. **10**(4): p. 273-84.
20. Chen, Z., et al., *Hypoxia-regulated microRNA-210 modulates mitochondrial function and decreases ISCU and COX10 expression.* Oncogene, 2010. **29**(30): p. 4362-8.
21. Castoldi, M., et al., *The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice.* J Clin Invest, 2011. **121**(4): p. 1386-96.
22. Esau, C., et al., *miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting.* Cell Metab, 2006. **3**(2): p. 87-98.
23. *Report of the American Institute of Nutrition ad hoc Committee on Standards for Nutritional Studies.* J Nutr, 1977. **107**(7): p. 1340-8.
24. Beard, J.L., C.S. Zhan, and D.E. Brigham, *Growth in iron-deficient rats.* Proc Soc Exp Biol Med, 1995. **209**(1): p. 65-72.
25. Davis, M.R., et al., *Enhanced expression of lipogenic genes may contribute to hyperglycemia and alterations in plasma lipids in response to dietary iron deficiency.* Genes Nutr, 2012.
26. Rebouche, C.J., C.L. Wilcox, and J.A. Widness, *Microanalysis of non-heme iron in animal tissues.* J Biochem Biophys Methods, 2004. **58**(3): p. 239-51.
27. Jagadeeswaran, G., et al., *Deep sequencing of small RNA libraries reveals dynamic regulation of conserved and novel microRNAs and microRNA-stars during silkworm development.* BMC Genomics, 2010. **11**: p. 52.
28. Van Nieuwerburgh, F., et al., *Quantitative bias in Illumina TruSeq and a novel post amplification barcoding strategy for multiplexed DNA and small RNA deep sequencing.* PLoS One, 2011. **6**(10): p. e26969.
29. Wang, W.C., et al., *miRExpress: analyzing high-throughput sequencing data for profiling microRNA expression.* BMC Bioinformatics, 2009. **10**: p. 328.
30. Altschul, S.F., et al., *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-10.
31. Dweep, H., et al., *miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes.* J Biomed Inform, 2011. **44**(5): p. 839-47.
32. Borel, M.J., J.L. Beard, and P.A. Farrell, *Hepatic glucose production and insulin sensitivity and responsiveness in iron-deficient anemic rats.* Am J Physiol, 1993. **264**(3 Pt 1): p. E380-90.
33. Davis, M.R., et al., *Comparisons of the iron deficient metabolic response in rats fed either an AIN-76 or AIN-93 based diet.* Nutr Metab (Lond), 2012. **9**(1): p. 95.
34. Borel, M.J., et al., *The impact of varying degrees of iron nutriture on several functional consequences of iron deficiency in rats.* J Nutr, 1991. **121**(5): p. 729-36.
35. Henao-Mejia, J., et al., *The MicroRNA miR-181 Is a Critical Cellular Metabolic Rheostat Essential for NKT Cell Ontogenesis and Lymphocyte Development and Homeostasis.* Immunity, 2013. **38**(5): p. 984-97.
36. Huang, X., Q.T. Le, and A.J. Giaccia, *MiR-210--micromanager of the hypoxia pathway.* Trends Mol Med, 2010. **16**(5): p. 230-7.
37. van Rooij, E., *The art of microRNA research.* Circ Res, 2011. **108**(2): p. 219-34.

38. Ouyang, Y.B., et al., *miR-181 targets multiple Bcl-2 family members and influences apoptosis and mitochondrial function in astrocytes.* Mitochondrion, 2012. **12**(2): p. 213-219.
39. Paradkar, P.N., et al., *Regulation of mitochondrial iron import through differential turnover of mitoferrin 1 and mitoferrin 2.* Mol Cell Biol, 2009. **29**(4): p. 1007-16.
40. Davies, K.J., et al., *Distinguishing effects of anemia and muscle iron deficiency on exercise bioenergetics in the rat.* Am J Physiol, 1984. **246**(6 Pt 1): p. E535-43.
41. Johnson, J.A., et al., *Muscle mitochondrial ultrastructure in exercise-trained iron-deficient rats.* J Appl Physiol, 1990. **68**(1): p. 113-8.
42. Corcoran, M.P., S. Lamon-Fava, and R.A. Fielding, *Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise.* Am J Clin Nutr, 2007. **85**(3): p. 662-77.
43. Turner, N., et al., *Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding.* Diabetologia, 2013. **56**(7): p. 1638-48.
44. Schreurs, M., F. Kuipers, and F.R. van der Leij, *Regulatory enzymes of mitochondrial beta-oxidation as targets for treatment of the metabolic syndrome.* Obes Rev, 2010. **11**(5): p. 380-8.

CHAPTER VII

CONCLUSIONS

The central hypothesis was that miRNA expression would be regulated in response to ID and that these changes would be associated with changes in the expression of target mRNA resulting in the homeostatic regulation of cellular iron metabolism. In order to test this hypothesis our primary aims were to (1) examine miRNA expression profile under iron-adequate and iron-restricted conditions in animals, (2) to identify differentially expressed miRNA, (3) to examine the potential targets of differentially expressed miRNA, and (4) to characterize the impact of miRNA expression on putative targets involved in iron metabolism. For each aim our findings were as follows:

Aim 1: To examine the miRNA expression profile of iron-adequate and iron-restricted animals.

Total hepatic RNA was successfully used to generate miRNA expression profiles from iron-adequate and iron-deficient animals. Some 30 million qualified reads were generated per animal, with 34% and 25% of those reads identified as either precursor or mature miRNA, respectively. More than 10 putative novel miRNA were also identified.

Aim 2: To identify miRNA which are differentially expressed in response to iron deficiency.

Bioinformatic analyses of the next-generation sequencing data produced in Aim 1 revealed more than 30 miRNA are differentially expressed in response to dietary iron deficiency.

Aim 3: To examine the potential targets of differentially expressed miRNA.

Hundreds of potential targets were predicted using multiple *in silico* target-prediction programs. The target list was then narrowed down to potential miR-targets that are known to be intimately involved in iron metabolism and homeostasis.

Aim 4: To characterize the impact of miRNA expression on putative targets involved in iron metabolism.

Through the use of *in vitro* reporter assays we were able to confirm that the miRNA do directly target mRNA with important roles in the maintenance of iron homeostasis.

The findings from each of these aims are supportive of our initial hypothesis that miRNA expression is altered in response to dietary iron deficiency, and that miRNA contribute to the homeostatic regulation of cellular iron metabolism. These results have provided insight into metabolic adaptation to iron deficiency and have demonstrated how miRNA contribute to the molecular coordination of iron homeostasis in a physiologic model of dietary iron deficiency.

Future directions

The field of miRNA research is growing at an exponential rate. Since the discovery of the first miRNA in worms in 1993, and the discovery of widespread species conservation of miRNA in the early 2000s, over 25,000 miRNA have been annotated and catalogued in nearly 200 different species [110]. Coinciding with the growth in miRNA numbers has been the growth in our knowledge and understanding of how miRNA themselves are regulated in addition to how they regulate target gene expression. Thus, miRNA research is fraught with challenges as the technology to study miRNA regulation and function has hinged upon our understanding of how they work, and has rapidly changed over time as our understanding has developed. Perhaps the most limiting factor in the current study was the lack of a high throughput technology capable of detecting the modest, but biologically significant, changes predicted to occur under non-pathologic environmental conditions, such as moderate iron deficiency [118, 131].

For instance, we chose to use next-generation sequencing (NGS) to create our miRNA profile. Though this technology is highly praised for its accuracy in distinguishing miRNA that are very similar in sequence, and its ability to detect novel miRNA, it is not without limitation; NGS cannot be used for absolute quantification, also the method of cDNA library preparation for NGS favors the capture of some miRNA over others, and NGS is not the most sensitive method for detecting small differences in miRNA expression [131, 146]. The other currently available technologies are also wrought with complications. For example, miRNA microarray analysis, is fairly high-throughput, but not as sensitive as qRT-PCR [146]. Likewise, qRT-PCR is very sensitive, but per sample is quite costly, and it cannot identify novel miRNA [146].

Thus, for our initial aim, NGS sequencing was the most appropriate currently available method, and even though NGS sequencing is not as sensitive for detecting differential miRNA expression as qRT-PCR, the results do tend to correlate very highly with results from downstream qRT-PCR analysis [147].

The fundamental determinant then for future studies will be deciding which of these limitations can we bypass to accurately test our hypothesis(es). Questions that still need to be answered include, what are the miRNA tissue-specific responses to ID, and what are the downstream effects of differential miRNA expression on target function and homeostatic iron regulation. Additionally, it may be of interest to investigate the potential for therapeutic miRNA targeting for treatment of diseases of iron metabolism. The findings from this work demonstrate that miRNA are regulated in response to dietary iron deficiency, and thus have laid the groundwork for future investigations by narrowing the list from the thousands of existing miRNA to a much smaller pool of miRNA that appear to be directly affected by alterations in iron status. A next logical step might then be to quantify these changes, and examine tissue specific differences in miRNA abundance utilizing qRT-PCR. Furthermore, a significant amount of work remains by way of continuing to identify and validate the iron-related targets of these differentially expressed miRNA.

Once targets have been validated, it would then be of great usefulness to interrogate the functional consequences of miRNA induced changes in iron-related gene expression. For instance in this study we found hepatic and skeletal muscle expression of miR-210 to be significantly elevated in response to dietary ID, but observed no changes in the expression of the miR-210 target, Iscu. Several explanations exist for these

observations, and should be the subject of future investigations. Perhaps with the moderate degree of ID elicited in this study, the effects on target gene expression were too modest to measure. Or, perhaps the tissues in question had already adapted to the state of ID, and we were measuring gene expression after equilibration had been achieved. Indeed, hepatic RNA binding activity of the cellular iron regulators IRP1 and IRP2 will begin to increase within as little as two days of placing animals on an iron restricted diet, and continue to increase for 10-14 days [95]. However after 2 weeks on an ID diet, IRP RNA binding activity tends to plateau [95]. As we only examined alterations in miRNA expression following 21 d of an ID diet it would be of great interest to assess the temporal regulation of miRNA and target mRNA expression throughout the progression of ID.

Also, because physiologic responses to miRNA regulation are often times quite modest, it may be necessary to over-express or inhibit miRNA expression in cells or in the whole-animal. For example, cell culture-based evidence is strongly suggestive that miR-210 induced repression of Iscu could elicit functional consequences on Fe-S cluster proteins [31, 32]. While our data does not refute this evidence, it does not strongly support it either since no measurable changes in Iscu expression were found. Also contesting this hypothesis is the fact that the effects of hypoxia and iron deficiency on IRP1 RNA binding activity in cultured rat liver hepatocytes elicit very different responses. This suggests that miR-210 target regulation may be stimulus dependent, and that potential confounding factors such as ceRNA may need to be investigated.

To interrogate this issue further, miR-210 mimics and inhibitors could be transfected into cells treated with or without hypoxia, and with or without the iron

chelator desferrioxamine, and Iscu protein abundance could be measured. The prediction would be that overexpression of miR-210 with either treatment should exacerbate the down-regulation of Iscu, while inhibition of miR-210 expression would decrease the effects on Iscu, or perhaps result in no down-regulation at all. If this prediction was met, further investigation of downstream effects on Fe-S cluster proteins, such as IRP1, would be warranted. If the hypothesis was not met, it would be relevant to begin to interrogate the factors that could interfere with miR-210/Iscu interaction, such as the simultaneous up regulation of other miR-210 targets that could potentially be titrating miR-210 away from Iscu in a stimulus-dependent manner.

In addition to advancing our understanding of the molecular coordination of iron homeostasis, many possibilities exist for exploiting both the therapeutic potential of miRNA for the treatment of diseases of iron metabolism and for the identification of plasma miRNA biomarkers that might be sensitive and timely indicators of changes in an individual's iron status. For example, mitochondrial iron overload is a prominent feature of human Fe-S cluster assembly disorders [46]. In this work we have demonstrated that the mitochondrial iron importer, Slc25a37, is targeted by the iron-regulated miR-181d. Future investigations might examine the potential for miR-181d to be used as a marker for mitochondrial iron overload. This would be difficult though because miR-181d would be predicted to possibly be down-regulated in an instance of mitochondrial iron overload. Therefore, it may be more warranted to investigate the therapeutic potential for miR-181d overexpression to protect against mitochondrial iron overload by repressing Slc25a37 and preventing mitochondrial iron import.

In this study we have also identified the key rate limiting enzyme in muscle β-oxidation, Cpt1a, as being directly targeted by miR-181d, therefore pharmacologic potential may also exist for the development of a miR-181d antagonist. In our previous studies and in this most recent work we have also observed decreased Cpt1A and Cpt1b expression in response to dietary ID [21, 148]. Chronic systemic inhibition of CPT1 has been found to cause intracellular lipid accumulation and insulin resistance in rats [149]. Interestingly, intramuscular lipid accumulation and insulin resistance have also been noted in rats subjected to an ID diet [21, 150]. Moreover, accumulation of triglycerides in skeletal muscle has also been noted in obese humans, and is thought to be a potential contributor to metabolic disruption and the development of insulin resistance [151, 152]. This may be of clinical significance because ID does not affect just under-nourished individuals, but over-nourished individuals as well, and the potential for ID to exacerbate metabolic disruption has not been fully investigated [153, 154]. Intriguingly, overexpression of Cpt1b has previously been shown to improve insulin sensitivity in rats fed a high-fat diet [155]. Thus, pharmacologic inhibition of miR-181d may have significant therapeutic potential as a stimulator of fatty acid oxidation in skeletal muscle.

The potential for miRNA to serve as therapeutic targets for the treatment of genetic disorders or for metabolic disease is quite exciting, though given the pleiotropic effects of altering miRNA function, it is important that we possess a more thorough understanding of miRNA biology and function. In light of the progress that has been made over the last decade regarding miRNA expression, biogenesis, processing, and function, nutritional scientists are well-positioned to examine the relationship between nutrient status and miRNA and provide insight into mechanisms coordinating nutrient-

gene interactions. These studies will further our understanding of the roles miRNA play in coordinating the molecular response to alterations in iron status, and provide fundamental insights into the understanding of how iron homeostasis is maintained and how alterations in iron sensing can lead to the development of disease.

REFERENCES

1. Ganz, T., *Hepcidin and its role in regulating systemic iron metabolism.* Hematology Am Soc Hematol Educ Program, 2006: p. 29-35, 507.
2. Collins, J.F., M. Wessling-Resnick, and M.D. Knutson, *Hepcidin regulation of iron transport.* J Nutr, 2008. **138**(11): p. 2284-8.
3. Anderson, C.P., et al., *Mammalian iron metabolism and its control by iron regulatory proteins.* Biochim Biophys Acta, 2012. **1823**(9): p. 1468-83.
4. Wallander, M.L., E.A. Leibold, and R.S. Eisenstein, *Molecular control of vertebrate iron homeostasis by iron regulatory proteins.* Biochim Biophys Acta, 2006. **1763**(7): p. 668-89.
5. Haile, D.J., et al., *Cellular regulation of the iron-responsive element binding protein: disassembly of the cubane iron-sulfur cluster results in high-affinity RNA binding.* Proc Natl Acad Sci U S A, 1992. **89**(24): p. 11735-9.
6. Guo, B., Y. Yu, and E.A. Leibold, *Iron regulates cytoplasmic levels of a novel iron-responsive element-binding protein without aconitase activity.* J Biol Chem, 1994. **269**(39): p. 24252-60.
7. Meyron-Holtz, E.G., et al., *Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis.* Embo J, 2004. **23**(2): p. 386-95.
8. Vashisht, A.A., et al., *Control of iron homeostasis by an iron-regulated ubiquitin ligase.* Science, 2009. **326**(5953): p. 718-21.
9. Iwai, K., et al., *Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: implications for degradation of oxidized proteins.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 4924-8.
10. Liew, Y.F. and N.S. Shaw, *Mitochondrial cysteine desulfurase iron-sulfur cluster S and aconitase are post-transcriptionally regulated by dietary iron in skeletal muscle of rats.* J Nutr, 2005. **135**(9): p. 2151-8.
11. Maguire, J.J., et al., *Effects of dietary iron deficiency of iron-sulfur proteins and bioenergetic functions of skeletal muscle mitochondria.* Biochim Biophys Acta, 1982. **679**(2): p. 210-20.
12. McLane, J.A., et al., *Physiological and biochemical effects of iron deficiency on rat skeletal muscle.* Am J Physiol, 1981. **241**(1): p. C47-54.
13. Dallman, P.R., *Biochemical basis for the manifestations of iron deficiency.* Annu Rev Nutr, 1986. **6**: p. 13-40.
14. Lill, R., *Function and biogenesis of iron-sulphur proteins.* Nature, 2009. **460**(7257): p. 831-8.

15. *Worldwide prevalence of anaemia 1993–2005 : WHO global database on anaemia*, E.M. Bruno de Benoist, Ines Egli and Mary Cogswell., Editor 2008, WHO.

16. Beard, J.L., *Iron biology in immune function, muscle metabolism and neuronal functioning.* J Nutr, 2001. **131**(2S-2): p. 568S-579S; discussion 580S.
17. Lynch, S.R., *Why nutritional iron deficiency persists as a worldwide problem.* J Nutr, 2011. **141**(4): p. 763S-768S.
18. Borel, M.J., J.L. Beard, and P.A. Farrell, *Hepatic glucose production and insulin sensitivity and responsiveness in iron-deficient anemic rats.* Am J Physiol, 1993. **264**(3 Pt 1): p. E380-90.
19. Davies, K.J., et al., *Distinguishing effects of anemia and muscle iron deficiency on exercise bioenergetics in the rat.* Am J Physiol, 1984. **246**(6 Pt 1): p. E535-43.
20. Farrell, P.A., J.L. Beard, and M. Druckenmiller, *Increased insulin sensitivity in iron-deficient rats.* J Nutr, 1988. **118**(9): p. 1104-9.
21. Davis, M.R., et al., *Enhanced expression of lipogenic genes may contribute to hyperglycemia and alterations in plasma lipids in response to dietary iron deficiency.* Genes Nutr, 2012.
22. Borel, M.J., et al., *The impact of varying degrees of iron nutriture on several functional consequences of iron deficiency in rats.* J Nutr, 1991. **121**(5): p. 729-36.
23. Gilles-Gonzalez, M.A. and G. Gonzalez, *Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses.* J Inorg Biochem, 2005. **99**(1): p. 1-22.
24. Yin, L., et al., *Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways.* Science, 2007. **318**(5857): p. 1786-9.
25. Barr, I., et al., *Ferric, not ferrous, heme activates RNA-binding protein DGCR8 for primary microRNA processing.* Proc Natl Acad Sci U S A, 2012. **109**(6): p. 1919-24.
26. Faller, M., et al., *Heme is involved in microRNA processing.* Nat Struct Mol Biol, 2007. **14**(1): p. 23-9.
27. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs.* Genome Res, 2009. **19**(1): p. 92-105.
28. Liu, X., K. Fortin, and Z. Mourelatos, *MicroRNAs: biogenesis and molecular functions.* Brain Pathol, 2008. **18**(1): p. 113-21.
29. Lagos-Quintana, M., et al., *Identification of tissue-specific microRNAs from mouse.* Curr Biol, 2002. **12**(9): p. 735-9.
30. Wienholds, E. and R.H. Plasterk, *MicroRNA function in animal development.* FEBS Lett, 2005. **579**(26): p. 5911-22.
31. Chan, S.Y., et al., *MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2.* Cell Metab, 2009. **10**(4): p. 273-84.
32. Yoshioka, Y., et al., *Micromanaging Iron Homeostasis: hypoxia-inducible micro-RNA-210 suppresses iron homeostasis-related proteins.* J Biol Chem, 2012. **287**(41): p. 34110-9.

33. Castoldi, M., et al., *The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice.* J Clin Invest, 2011. **121**(4): p. 1386-96.
34. Esau, C., et al., *miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting.* Cell Metab, 2006. **3**(2): p. 87-98.
35. Ackrell, B.A., et al., *Effect of iron deficiency on succinate- and NADH-ubiquinone oxidoreductases in skeletal muscle mitochondria.* J Biol Chem, 1984. **259**(16): p. 10053-9.
36. Azevedo, J.L., Jr., et al., *Reciprocal changes of muscle oxidases and liver enzymes with recovery from iron deficiency.* Am J Physiol, 1989. **256**(3 Pt 1): p. E401-5.
37. Castoldi, M. and M.U. Muckenthaler, *Regulation of iron homeostasis by microRNAs.* Cell Mol Life Sci, 2012.
38. Poskitt, E.M., *Early history of iron deficiency.* Br J Haematol, 2003. **122**(4): p. 554-62.
39. Davies, D.T., *Simple achlorhydic anaemia.* Lancet, 1931. **2**: p. 385-391.
40. Witts, L.J., *Chronic Microcytic Anaemia.* Br Med J, 1931. **2**(3697): p. 883-8.
41. Hou, W., et al., *The let-7 microRNA enhances heme oxygenase-1 by suppressing Bach1 and attenuates oxidant injury in human hepatocytes.* Biochim Biophys Acta, 2012. **1819**(11-12): p. 1113-22.
42. Stoltzfus, R.J., *Iron deficiency: global prevalence and consequences.* Food Nutr Bull, 2003. **24**(4 Suppl): p. S99-103.
43. Hentze, M.W., M.U. Muckenthaler, and N.C. Andrews, *Balancing acts: molecular control of mammalian iron metabolism.* Cell, 2004. **117**(3): p. 285-97.
44. Cammack, R., Wrigglesworth, J. M., & Baum, H. (1990). Iron-dependent enzymes in mammalian systems. Iron transport and storage, 17-40., *Cammack, R., Wrigglesworth, J. M., & Baum, H. (1990). Iron-dependent enzymes in mammalian systems. Iron transport and storage, 17-40.*, in *Iron transport and storage*. 1990.
45. Sheftel, A., O. Stehling, and R. Lill, *Iron-sulfur proteins in health and disease.* Trends Endocrinol Metab, 2010. **21**(5): p. 302-14.
46. Rouault, T.A. and W.H. Tong, *Iron-sulfur cluster biogenesis and human disease.* Trends Genet, 2008. **24**(8): p. 398-407.
47. Winterbourn, C.C., *Toxicity of iron and hydrogen peroxide: the Fenton reaction.* Toxicol Lett, 1995. **82-83**: p. 969-74.
48. Zecca, L., et al., *Iron, brain ageing and neurodegenerative disorders.* Nat Rev Neurosci, 2004. **5**(11): p. 863-73.
49. Cai, L. and Y.J. Kang, *Oxidative stress and diabetic cardiomyopathy: a brief review.* Cardiovasc Toxicol, 2001. **1**(3): p. 181-93.
50. Liu, Q., et al., *Role of iron deficiency and overload in the pathogenesis of diabetes and diabetic complications.* Curr Med Chem, 2009. **16**(1): p. 113-29.
51. de Valk, B. and J.J. Marx, *Iron, atherosclerosis, and ischemic heart disease.* Arch Intern Med, 1999. **159**(14): p. 1542-8.
52. Pietrangelo, A., *Hereditary hemochromatosis.* Biochim Biophys Acta, 2006. **1763**(7): p. 700-10.
53. Andrews, N.C., *Iron metabolism: iron deficiency and iron overload.* Annu Rev Genomics Hum Genet, 2000. **1**: p. 75-98.

54. Vujic Spasic, M., et al., *Hfe acts in hepatocytes to prevent hemochromatosis.* Cell Metab, 2008. **7**(2): p. 173-8.
55. Camaschella, C., et al., *Juvenile and adult hemochromatosis are distinct genetic disorders.* Eur J Hum Genet, 1997. **5**(6): p. 371-5.
56. Miret, S., R.J. Simpson, and A.T. McKie, *Physiology and molecular biology of dietary iron absorption.* Annu Rev Nutr, 2003. **23**: p. 283-301.
57. Duffy, S.P., et al., *The Fowler syndrome-associated protein FLVCR2 is an importer of heme.* Mol Cell Biol, 2010. **30**(22): p. 5318-24.
58. Qiu, A., et al., *Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption.* Cell, 2006. **127**(5): p. 917-28.
59. Shayeghi, M., et al., *Identification of an intestinal heme transporter.* Cell, 2005. **122**(5): p. 789-801.
60. Andrews, N.C., *Forging a field: the golden age of iron biology.* Blood, 2008. **112**(2): p. 219-30.
61. Gunshin, H., et al., *Cybrd1 (duodenal cytochrome b) is not necessary for dietary iron absorption in mice.* Blood, 2005. **106**(8): p. 2879-83.
62. Hentze, M.W., et al., *Two to tango: regulation of Mammalian iron metabolism.* Cell, 2010. **142**(1): p. 24-38.
63. Andrews, N.C. and P.J. Schmidt, *Iron homeostasis.* Annu Rev Physiol, 2007. **69**: p. 69-85.
64. Zoller, H., et al., *Expression of the duodenal iron transporters divalent-metal transporter 1 and ferroportin 1 in iron deficiency and iron overload.* Gastroenterology, 2001. **120**(6): p. 1412-9.
65. Dunn, L.L., Y. Suryo Rahmanto, and D.R. Richardson, *Iron uptake and metabolism in the new millennium.* Trends Cell Biol, 2007. **17**(2): p. 93-100.
66. Eisenstein, R.S., *Iron regulatory proteins and the molecular control of mammalian iron metabolism.* Annu Rev Nutr, 2000. **20**: p. 627-62.
67. West, A.P., Jr., et al., *Comparison of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the hereditary hemochromatosis protein HFE.* J Biol Chem, 2000. **275**(49): p. 38135-8.
68. Camaschella, C., *Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders.* Blood, 2005. **106**(12): p. 3710-7.
69. Dautry-Varsat, A., A. Ciechanover, and H.F. Lodish, *pH and the recycling of transferrin during receptor-mediated endocytosis.* Proc Natl Acad Sci U S A, 1983. **80**(8): p. 2258-62.
70. Harrison, P.M. and P. Arosio, *The ferritins: molecular properties, iron storage function and cellular regulation.* Biochim Biophys Acta, 1996. **1275**(3): p. 161-203.
71. Arosio, P. and S. Levi, *Cytosolic and mitochondrial ferritins in the regulation of cellular iron homeostasis and oxidative damage.* Biochim Biophys Acta, 2010. **1800**(8): p. 783-92.
72. Cozzi, A., et al., *Analysis of the biologic functions of H- and L-ferritins in HeLa cells by transfection with siRNAs and cDNAs: evidence for a proliferative role of L-ferritin.* Blood, 2004. **103**(6): p. 2377-83.
73. Anderson, G.J., et al., *Regulation of systemic iron homeostasis: how the body responds to changes in iron demand.* Biometals, 2007. **20**(3-4): p. 665-74.

74. Knutson, M.D., et al., *Iron release from macrophages after erythrophagocytosis is up-regulated by ferroportin 1 overexpression and down-regulated by hepcidin.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1324-8.
75. Andrews, N.C., *Molecular control of iron metabolism.* Best Pract Res Clin Haematol, 2005. **18**(2): p. 159-69.
76. Nicolas, G., et al., *The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation.* J Clin Invest, 2002. **110**(7): p. 1037-44.
77. De Domenico, I., et al., *The molecular mechanism of hepcidin-mediated ferroportin down-regulation.* Mol Biol Cell, 2007. **18**(7): p. 2569-78.
78. Goswami, T. and N.C. Andrews, *Hereditary hemochromatosis protein, HFE, interaction with transferrin receptor 2 suggests a molecular mechanism for mammalian iron sensing.* J Biol Chem, 2006. **281**(39): p. 28494-8.
79. D'Alessio, F., M.W. Hentze, and M.U. Muckenthaler, *The hemochromatosis proteins HFE, TfR2, and HJV form a membrane-associated protein complex for hepcidin regulation.* J Hepatol, 2012. **57**(5): p. 1052-60.
80. Babitt, J.L., et al., *Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance.* J Clin Invest, 2007. **117**(7): p. 1933-9.
81. Wrighting, D.M. and N.C. Andrews, *Interleukin-6 induces hepcidin expression through STAT3.* Blood, 2006. **108**(9): p. 3204-9.
82. Nicolas, G., et al., *Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice.* Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8780-5.
83. Nicolas, G., et al., *Severe iron deficiency anemia in transgenic mice expressing liver hepcidin.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4596-601.
84. De Domenico, I., D.M. Ward, and J. Kaplan, *Hepcidin regulation: ironing out the details.* J Clin Invest, 2007. **117**(7): p. 1755-8.
85. Finberg, K.E., et al., *Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA).* Nat Genet, 2008. **40**(5): p. 569-71.
86. Eisenstein, R.S. and K.P. Blemings, *Iron regulatory proteins, iron responsive elements and iron homeostasis.* J Nutr, 1998. **128**(12): p. 2295-8.
87. Rouault, T.A., et al., *Structural relationship between an iron-regulated RNA-binding protein (IRE-BP) and aconitase: functional implications.* Cell, 1991. **64**(5): p. 881-3.
88. Salahudeen, A.A., et al., *An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis.* Science, 2009. **326**(5953): p. 722-6.
89. Pantopoulos, K., G. Weiss, and M.W. Hentze, *Nitric oxide and oxidative stress (H2O2) control mammalian iron metabolism by different pathways.* Mol Cell Biol, 1996. **16**(7): p. 3781-8.
90. Clarke, S.L., et al., *Iron-responsive degradation of iron-regulatory protein 1 does not require the Fe-S cluster.* Embo J, 2006. **25**(3): p. 544-53.
91. Brown, N.M., et al., *Novel role of phosphorylation in Fe-S cluster stability revealed by phosphomimetic mutations at Ser-138 of iron regulatory protein 1.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15235-40.

92. Schalinske, K.L., et al., *The iron-sulfur cluster of iron regulatory protein 1 modulates the accessibility of RNA binding and phosphorylation sites.* Biochemistry, 1997. **36**(13): p. 3950-8.
93. Schalinske, K.L. and R.S. Eisenstein, *Phosphorylation and activation of both iron regulatory proteins 1 and 2 in HL-60 cells.* J Biol Chem, 1996. **271**(12): p. 7168-76.
94. Chen, O.S., et al., *Dietary iron intake rapidly influences iron regulatory proteins, ferritin subunits and mitochondrial aconitase in rat liver.* J Nutr, 1998. **128**(3): p. 525-35.
95. Ross, K.L. and R.S. Eisenstein, *Iron deficiency decreases mitochondrial aconitase abundance and citrate concentration without affecting tricarboxylic acid cycle capacity in rat liver.* J Nutr, 2002. **132**(4): p. 643-51.
96. Sanchez, M., et al., *Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency.* Nat Struct Mol Biol, 2007. **14**(5): p. 420-6.
97. Meyron-Holtz, E.G., M.C. Ghosh, and T.A. Rouault, *Mammalian tissue oxygen levels modulate iron-regulatory protein activities in vivo.* Science, 2004. **306**(5704): p. 2087-90.
98. Wang, G.L. and G.L. Semenza, *Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction.* Blood, 1993. **82**(12): p. 3610-5.
99. Anderson, S.A., et al., *The IRP1-HIF-2alpha axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption.* Cell Metab, 2013. **17**(2): p. 282-90.
100. Davis, M.R., et al., *Hypoxia inducible factor-2 alpha is translationally repressed in response to dietary iron deficiency in Sprague-Dawley rats.* J Nutr, 2011. **141**(9): p. 1590-6.
101. Kispal, G., et al., *The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins.* EMBO J, 1999. **18**(14): p. 3981-9.
102. Pondarre, C., et al., *The mitochondrial ATP-binding cassette transporter Abcb7 is essential in mice and participates in cytosolic iron-sulfur cluster biogenesis.* Hum Mol Genet, 2006. **15**(6): p. 953-64.
103. Galy, B., et al., *Iron regulatory proteins secure mitochondrial iron sufficiency and function.* Cell Metab, 2010. **12**(2): p. 194-201.
104. Chen, O.S., K.L. Schalinske, and R.S. Eisenstein, *Dietary iron intake modulates the activity of iron regulatory proteins and the abundance of ferritin and mitochondrial aconitase in rat liver.* J Nutr, 1997. **127**(2): p. 238-48.
105. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**(5): p. 843-54.
106. Reinhart, B.J., et al., *The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans.* Nature, 2000. **403**(6772): p. 901-6.
107. Pasquinelli, A.E., et al., *Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA.* Nature, 2000. **408**(6808): p. 86-9.
108. Lagos-Quintana, M., et al., *Identification of novel genes coding for small expressed RNAs.* Science, 2001. **294**(5543): p. 853-8.

109. Lau, N.C., et al., *An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans.* Science, 2001. **294**(5543): p. 858-62.
110. Kozomara, A. and S. Griffiths-Jones, *miRBase: integrating microRNA annotation and deep-sequencing data.* Nucleic Acids Res, 2011. **39**(Database issue): p. D152-7.
111. Ambros, V., et al., *A uniform system for microRNA annotation.* RNA, 2003. **9**(3): p. 277-9.
112. Griffiths-Jones, S., et al., *miRBase: microRNA sequences, targets and gene nomenclature.* Nucleic Acids Res, 2006. **34**(Database issue): p. D140-4.
113. *miRBase: What do the names/identifiers mean?* 2012 [cited 2013 April 5, 2013]; Available from: http://www.mirbase.org/help/nomenclature.shtml.
114. Rodriguez, A., et al., *Identification of mammalian microRNA host genes and transcription units.* Genome Res, 2004. **14**(10A): p. 1902-10.
115. Ambros, V., *The functions of animal microRNAs.* Nature, 2004. **431**(7006): p. 350-5.
116. Slezak-Prochazka, I., et al., *MicroRNAs, macrocontrol: regulation of miRNA processing.* Rna, 2010. **16**(6): p. 1087-95.
117. Kim, V.N., J. Han, and M.C. Siomi, *Biogenesis of small RNAs in animals.* Nat Rev Mol Cell Biol, 2009. **10**(2): p. 126-39.
118. Huntzinger, E. and E. Izaurralde, *Gene silencing by microRNAs: contributions of translational repression and mRNA decay.* Nat Rev Genet, 2011. **12**(2): p. 99-110.
119. Ender, C. and G. Meister, *Argonaute proteins at a glance.* J Cell Sci, 2010. **123**(Pt 11): p. 1819-23.
120. Kawamata, T. and Y. Tomari, *Making RISC.* Trends Biochem Sci, 2010. **35**(7): p. 368-76.
121. Grimson, A., et al., *MicroRNA targeting specificity in mammals: determinants beyond seed pairing.* Mol Cell, 2007. **27**(1): p. 91-105.
122. Lytle, J.R., T.A. Yario, and J.A. Steitz, *Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9667-72.
123. Okamura, K., et al., *The regulatory activity of microRNA* species has substantial influence on microRNA and 3′ UTR evolution.* Nat Struct Mol Biol, 2008. **15**(4): p. 354-63.
124. Yang, J.S., et al., *Widespread regulatory activity of vertebrate microRNA* species.* Rna, 2011. **17**(2): p. 312-26.
125. Li, Y., et al., *Iron homeostasis regulates the activity of the microRNA pathway through poly(C)-binding protein 2.* Cell Metab, 2012. **15**(6): p. 895-904.
126. Calin, G.A. and C.M. Croce, *MicroRNA-cancer connection: the beginning of a new tale.* Cancer Res, 2006. **66**(15): p. 7390-4.
127. Rottiers, V. and A.M. Naar, *MicroRNAs in metabolism and metabolic disorders.* Nat Rev Mol Cell Biol, 2012. **13**(4): p. 239-50.
128. Pasquinelli, A.E., *MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship.* Nat Rev Genet, 2012. **13**(4): p. 271-82.
129. Saito, T. and P. Saetrom, *MicroRNAs--targeting and target prediction.* N Biotechnol, 2010. **27**(3): p. 243-9.

130. Thomas, M., J. Lieberman, and A. Lal, *Desperately seeking microRNA targets.* Nat Struct Mol Biol, 2010. **17**(10): p. 1169-74.
131. van Rooij, E., *The art of microRNA research.* Circ Res, 2011. **108**(2): p. 219-34.
132. Hu, W. and J. Coller, *What comes first: translational repression or mRNA degradation? The deepening mystery of microRNA function.* Cell Res, 2012. **22**(9): p. 1322-4.
133. Karreth, F.A., et al., *In vivo identification of tumor- suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma.* Cell, 2011. **147**(2): p. 382-95.
134. Andolfo, I., et al., *Regulation of divalent metal transporter 1 (DMT1) non-IRE isoform by the microRNA Let-7d in erythroid cells.* Haematologica, 2010. **95**(8): p. 1244-52.
135. Schaar, D.G., et al., *miR-320 targets transferrin receptor 1 (CD71) and inhibits cell proliferation.* Exp Hematol, 2009. **37**(2): p. 245-55.
136. Liao, Y., X. Du, and B. Lonnerdal, *miR-214 regulates lactoferrin expression and pro-apoptotic function in mammary epithelial cells.* J Nutr, 2010. **140**(9): p. 1552-6.
137. Liao, Y. and B. Lonnerdal, *miR-584 mediates post-transcriptional expression of lactoferrin receptor in Caco-2 cells and in mouse small intestine during the perinatal period.* Int J Biochem Cell Biol, 2010. **42**(8): p. 1363-9.
138. Sangokoya, C., J.F. Doss, and J.T. Chi, *Iron-Responsive miR-485-3p Regulates Cellular Iron Homeostasis by Targeting Ferroportin.* PLoS Genet, 2013. **9**(4): p. e1003408.
139. Shpyleva, S.I., et al., *Role of ferritin alterations in human breast cancer cells.* Breast Cancer Res Treat, 2011. **126**(1): p. 63-71.
140. Ryu, M.S., et al., *Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis.* Proc Natl Acad Sci U S A, 2011. **108**(52): p. 20970-5.
141. Hou, W., et al., *MicroRNA-196 represses Bach1 protein and hepatitis C virus gene expression in human hepatoma cells expressing hepatitis C viral proteins.* Hepatology, 2010. **51**(5): p. 1494-504.
142. Hintze, K.J., et al., *Bach1 repression of ferritin and thioredoxin reductase1 is heme-sensitive in cells and in vitro and coordinates expression with heme oxygenase1, beta-globin, and NADP(H) quinone (oxido) reductase1.* J Biol Chem, 2007. **282**(47): p. 34365-71.
143. Siaj, R., et al., *Longitudinal analysis of serum miR-122 in a rat model of Wilson's disease.* Hepatol Int, 2012. **6**(4): p. 770-777.
144. Listowski, M.A., et al., *microRNAs: fine tuning of erythropoiesis.* Cell Mol Biol Lett, 2013. **18**(1): p. 34-46.
145. Azzouzi, I., et al., *MicroRNA-96 directly inhibits gamma-globin expression in human erythropoiesis.* PLoS One, 2011. **6**(7): p. e22838.
146. Pritchard, C.C., H.H. Cheng, and M. Tewari, *MicroRNA profiling: approaches and considerations.* Nat Rev Genet, 2012. **13**(5): p. 358-69.
147. Tian, G., et al., *Sequencing bias: comparison of different protocols of microRNA library construction.* BMC Biotechnol, 2010. **10**: p. 64.

148. Davis, M.R., et al., *Comparisons of the iron deficient metabolic response in rats fed either an AIN-76 or AIN-93 based diet.* Nutr Metab (Lond), 2012. **9**(1): p. 95.
149. Dobbins, R.L., et al., *Prolonged inhibition of muscle carnitine palmitoyltransferase-1 promotes intramyocellular lipid accumulation and insulin resistance in rats.* Diabetes, 2001. **50**(1): p. 123-30.
150. Johnson, J.A., et al., *Muscle mitochondrial ultrastructure in exercise-trained iron-deficient rats.* J Appl Physiol, 1990. **68**(1): p. 113-8.
151. Corcoran, M.P., S. Lamon-Fava, and R.A. Fielding, *Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise.* Am J Clin Nutr, 2007. **85**(3): p. 662-77.
152. Schreurs, M., F. Kuipers, and F.R. van der Leij, *Regulatory enzymes of mitochondrial beta-oxidation as targets for treatment of the metabolic syndrome.* Obes Rev, 2010. **11**(5): p. 380-8.
153. Lecube, A., et al., *Iron deficiency in obese postmenopausal women.* Obesity (Silver Spring), 2006. **14**(10): p. 1724-30.
154. Pinhas-Hamiel, O., et al., *Greater prevalence of iron deficiency in overweight and obese children and adolescents.* Int J Obes Relat Metab Disord, 2003. **27**(3): p. 416-8.
155. Bruce, C.R., et al., *Overexpression of carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high-fat diet-induced insulin resistance.* Diabetes, 2009. **58**(3): p. 550-8

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