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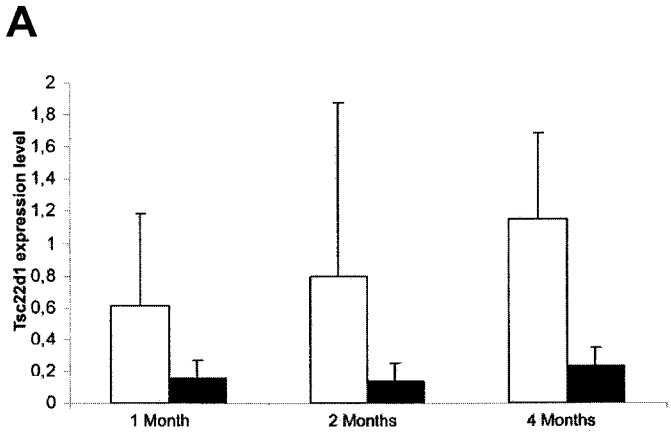

(57) Abstract: The present invention identifies genes having expression profiles correlated with and predictive of iron-related disorders. The present invention includes methods for diagnosing iron-related disorders and monitoring the progression of or efficacy of treatment of iron-related disorders by determining the expression or activity of these genes or their encoded polypeptides. In addition, the present invention includes compositions and methods of modulating these genes and treating or preventing iron-related disorders.

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COMPOSITIONS AND METHODS FOR THE DIAGNOSIS AND TREATMENT OF IRON-RELATED DISORDERS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 60/876,718 filed December 22, 2006, where this provisional application is incorporated herein by reference in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 980057_447PC_SEQUENCE_LISTING.txt. The text file is 886 KB, was created on December 19, 2007, and is being submitted electronically via EFS-Web to the U.S. PCT Receiving Office, concurrent with the filing of the specification.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is related to the diagnosis and treatment of iron-related disorders, including iron deficiency, anemia of inflammation, and iron overload. More particularly, the present invention is related to the identification of genes having altered expression in iron-related disorders, and methods and compounds for modulating the expression or activity of these genes and their encoded polypeptides.

Description of the Related Art

Iron homeostasis is critical for the normal function of the body. Both iron deficiency and iron overload can have deleterious effect on this tenuous balance, resulting in a variety of iron-related disorders. A critical function for iron is that it participates in hemoglobin production. Therefore, too little iron results in iron-deficient anemia. Iron overload can be due to an inappropriate increase in intestinal iron absorption. The increase can result in the depositing of iron in the liver, pancreas, heart, pituitary, and other organs, leading to tissue damage and impairment of normal function of those organs. Iron overload results in a variety of

disorders, including cardiomyopathy, cirrhosis, diabetes, hypogonadism, and liver disease. In contrast, Anemia of Inflammation (AI), also known as Anemia of Chronic Disease, is an acquired disorder characterized by a maldistribution of iron that is associated with a variety of disorders, including infection, malignancy and chronic inflammation.

Iron-related disorders can be difficult to diagnose. Current procedures fail to comprehensively test for iron disorders, because the symptoms are often vague and similar to those associated with other conditions. In addition, the severe effects of iron-related diseases tend not to appear immediately. The available preliminary screenings are often inconclusive, as the current tests have only modest predictive value. For example, current methodologies for diagnosing iron overload include biochemical testing and molecular DNA analyses. However, biochemical markers of iron overload may only appear late in the course of the disease, and the DNA testing (*e.g.*, hemochromatosis gene (HFE) mutation detection) is not a reliable predictor of which patients will overload iron. Furthermore, a negative biochemical screen does not preclude the disease, as iron overload is an age-dependent disorder and may only manifest at a later date.

AI presents additional diagnostic challenges, as AI can resemble the anemia seen in true iron deficiency, although the two conditions are treated differently. Furthermore, true iron deficiency often accompanies AI. If left undiagnosed, AI can result in inappropriate treatments in an effort to correct the anemia.

Many patients suffering from iron-related disorders have serious health problems that require multiple treatments, such as repeated blood transfusion, iron infusions, iron injections, or iron-chelation therapy to remove the extra iron. Some may need erythropoeitin (EPO) to stimulate bone marrow production of red cells. Some diseases can be chronic, where reaching iron balance is a challenge, such as, for example, kidney disease, cancer, thalassemia, sickle cell disease, CDA II (HEMPAS), inherited sideroblastic anemia, myelodysplasia (MDS), porphyria cutanea tarda (PCT), hereditary hemorrhagic telangiectasia (HHT), AIDS, Crohn's, celiac disease, and autoimmune hemolytic anemias.

The current difficulties in diagnosing iron-related disorders can cause delays in administering the much-needed treatment. Clearly, therefore, the need exists for reliable and non-invasive diagnostic tests for the detection of iron-related disorders in patients, as well as a diagnostic test that would clearly differentiate AI

from true iron deficiency. In addition, there remains an unmet need for new methods of treating iron-related disorders.

BRIEF SUMMARY OF THE INVENTION

The present invention provides diagnostic, prognostic, and therapeutic methods, based upon the identification of genes and polypeptides having altered expression or activity in iron-related disorders.

In a first embodiment, the present invention provides a method for determining whether a patient has an iron-related disorder, said method comprising measuring in a patient or in a biological sample obtained from said patient the level of expression of one or more polynucleotides comprising a sequence set forth in SEQ ID NOs: 98-178, or a homolog or ortholog thereof, wherein an altered level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder. In particular embodiments, the method comprises measuring the level of expression of two or more polynucleotides. In one embodiment, the polynucleotides are mRNAs. In certain embodiments, the level of expression of said one or more polynucleotides is greater than the normal control level. In other embodiments, the level of expression of said one or more polynucleotides is less than the normal control level. In some embodiments, the level of one or more polynucleotides is greater than a normal control, and the level of one or more other polynucleotides is less than normal control.

In a related embodiment, the present invention includes a method for determining whether a patient has an iron-related disorder, said method comprising determining the presence or absence of a mutation or polymorphism in one or more of a patient's genes encoding polypeptides having a sequence set forth in SEQ ID NOs: 179-253, or a homolog or ortholog thereof, wherein the presence of said mutation or polymorphism indicates that said patient has an iron-related disorder.

In another related embodiment, the present invention provides a method for determining whether a patient has an iron-related disorder, said method comprising measuring in said patient or a biological sample obtained from said patient the level of biological activity of one or more polypeptides having an amino acid sequence set forth in SEQ ID NOs: 179-253, or a homolog or ortholog thereof, wherein an altered level in said biological activity, relative to a normal control level, indicates that said patient has an iron-related disorder.

In a further embodiment, the present invention provides a method for determining whether a patient has an iron-related disorder, said method comprising measuring in said patient or a biological sample obtained from said patient the level of expression of one or more polypeptides having an amino acid sequence set forth in SEQ ID NOs: 179-253, or a homolog or ortholog thereof, wherein an altered level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder.

The present invention, in another embodiment, also provides a method for determining whether a patient has an iron-related disorder related to iron overload, said method comprising measuring the level of expression of one or more polynucleotides comprising a sequence set forth in SEQ ID NOs: 98-110, 124-140, 148, 149, and 166, or a homolog or ortholog thereof, or of one or more polypeptides comprising a sequence set forth in SEQ ID NOs: 179-189, 203-215, 223, 224 and 241, wherein an increased level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder related to iron overload.

In a related embodiment, the present invention includes a method for determining whether a patient has an iron-related disorder related to iron deficiency, said method comprising measuring the level of expression of one or more polynucleotides comprising a sequence set forth in SEQ ID NOs: 98-110, 124-140, 148, 149, and 166, or a homolog or ortholog thereof, or of one or more polypeptides comprising a sequence set forth in SEQ ID NOs: 179-189, 203-215, 223, 224 and 241, or a homolog or ortholog thereof, wherein a decreased level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder related to iron overload.

In another embodiment, the present invention includes a method for determining whether a patient has an iron-related disorder related to iron overload, said method comprising measuring the level of expression of one or more polynucleotides comprising a sequence set forth in SEQ ID NOs: 111-123, 143, and 151, or a homolog or ortholog thereof, or of one or more polypeptides comprising a sequence set forth in SEQ ID NOs: 190-202, 216-218, and 226, wherein a decreased level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder related to iron overload.

In yet another related embodiment, the present invention includes a method for determining whether a patient has an iron-related disorder related to iron deficiency, said method comprising measuring the level of expression of one or more polynucleotides comprising a sequence set forth in SEQ ID NOs: 111-123,

143, and 151, or a homolog or ortholog thereof, or of one or more polypeptides comprising a sequence set forth in SEQ ID NOs: 190-202, 216-218, and 226, or a homolog or ortholog thereof, wherein an increased level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder related to iron deficiency.

The present invention further provides, in another embodiment, a method for determining if a treatment for an iron-related disorder is efficacious, comprising: measuring in a patient or in a biological sample obtained from said patient, at a first time point prior to treatment, the level of expression or activity of one or more polynucleotides comprising a sequence set forth in SEQ ID NOs: 98-178, wherein said level of expression is altered by a first amount relative to a normal control level; and measuring in a patient or in a biological sample obtained from said patient, at a second time point following treatment, the level of expression or activity of the one or more polypeptides of step (a), wherein said level of expression is altered by a second amount relative to a normal control level, wherein if the first amount is greater than the second amount, it indicates that the treatment is efficacious.

In another embodiment, the present invention provides a method for monitoring the progression of an iron-related disorder in a patient, comprising: measuring in a patient diagnosed with or suspected of having an iron-related disorder, or in a biological sample obtained from said patient, at a first time point, a level of expression or activity of one or more polynucleotides comprising a sequence set forth in SEQ ID NOs: 98-178 and determining a first relative value by which the level is altered as compared to a normal control level; measuring in the patient or in a biological sample obtained from said patient, at a later second time point, a level of expression or activity of the one or more polypeptides of step (a) and determining a second relative value by which the level is altered as compared to a normal control level, wherein if the first relative value is less than the second relative value, it indicates that the iron-related disorder is progressing, and if the first relative value is greater than the second relative value, it indicates that the iron-related disorder is in remission.

In yet a further related embodiment, the present invention provides a method for identifying a compound for the treatment or prevention of an iron-related disorder, said method comprising: contacting a cell expressing a polynucleotide encoding a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 179-253, or a variant or fragment thereof, with a candidate compound; and measuring the level of expression or a biological activity of said

polynucleotide or polypeptide, wherein an altered level of expression or biological activity of said polynucleotide or polypeptide, relative to the level in a cell not contacted with said compound, indicates that said candidate compound is useful for the treatment of an iron-related disorder.

In yet another embodiment, the present invention includes a method for identifying a compound for the treatment or prevention of an iron-related disorder, said method comprising contacting a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253, or a variant or fragment thereof, with a candidate compound; and determining whether said candidate compound binds to said polypeptide, wherein binding of said candidate compound to said polypeptide identifies said candidate compound as a compound useful for the treatment or prevention of an iron-related disorder.

In a further related embodiment, the present invention includes a method for identifying a compound for the treatment of an iron-related disorder, said method comprising administering a candidate compound to a transgenic animal expressing a transgene encoding a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253, or a variant or fragment thereof, and determining whether said candidate compound alters a biological activity or the level of expression of said transgene or polypeptide, wherein an alteration in the biological activity or level of expression of said transgene or polypeptide identifies said candidate compound as a compound useful for the treatment of an iron-related disorder.

In another embodiment, the present invention includes a method for identifying a compound for the treatment of a iron-related disorder, said method comprising the steps of administering a candidate compound to a transgenic animal expressing a transgene encoding a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253, or a variant or fragment thereof, said animal having an iron-related disorder; and determining whether said candidate compound alleviates said iron-related disorder.

In a further embodiment, the present invention includes a method for identifying a compound for the treatment of an iron-related disorder, said method comprising the steps of administering a candidate compound to a animal comprising a mutation in a gene encoding a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253, or an ortholog thereof, and determining whether said candidate compound alters a biological activity or the level of expression of said gene or polypeptide, wherein an alteration in the biological activity or level of expression of said polypeptide identifies said

candidate compound as a compound useful for the treatment of an iron-related disorder.

In another related embodiment, the present invention includes a method for identifying a compound for the treatment of an iron-related disorder, said method comprising the steps of administering a candidate compound to a animal comprising a mutation in a gene encoding a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253, or an ortholog thereof, said animal having an iron-related disorder; and determining whether said candidate compound alleviates said iron-related disorder.

The present invention further provides an animal model of an iron-related disorder, wherein said animal model is a non-human mammal comprising a transgene that expresses a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253, or a fragment or variant thereof, wherein said non-human mammal exhibits one or more symptoms of an iron-related disorder.

The present invention, in another embodiment, provides an animal model of an iron-related disorder, wherein said animal model is a non-human mammal comprising a mutation in a gene encoding a polypeptide having an amino acid sequence set forth in SEQ ID NOs: SEQ ID NOs: 49-97 and 179-253, or an ortholog thereof, wherein said non-human mammal exhibits one or more symptoms of a iron-related disorder.

In a further embodiment, the present invention includes a cell isolated from the non-human mammal of the present invention.

In yet another embodiment, the present invention includes a kit for determining the presence of an iron-related disorder in a patient, wherein said kit comprises one or more compound that specifically bind to a polypeptide having a sequence set forth in SEQ ID NOs: 179-253 or specifically bind to a polynucleotide that encodes a polypeptide having a sequence set forth in SEQ ID NOs: 179-253. In particular embodiments, said kit comprises an array of compounds that specifically bind to polypeptides having a sequence set forth in SEQ ID NOs: 179-253 or specifically bind to polynucleotides that encode polypeptides having a sequence set forth in SEQ ID NOs: 179-253. In one embodiment, said one or more compounds are labeled. In particular embodiments, said compounds are oligonucleotides. In one embodiment, said polypeptides are secreted.

In another embodiment, the present invention provides a method of treating or preventing an iron-related disorder in a patient, said method comprising administering to said patient a nucleic acid molecule encoding a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253, or a

variant or fragment thereof. In particular embodiments, the method comprises administering to said patient an expression vector comprising a nucleic acid molecule operably linked to a promoter, said nucleic acid molecule encodes a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253, or a variant or fragment thereof.

In another embodiment, the present invention provides a method of treating or preventing an iron-related disorder in a patient, said method comprising administering to said patient a compound that modulates a biological activity or expression of a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 179-253. In one embodiment, the compound is a nucleic acid molecule comprising a portion of a polynucleotide sequence set forth in SEQ ID NOs: 1-48 and 98-178 or a complement thereof. In particular embodiments, the nucleic acid molecule is selected from the group consisting of: viruses, plasmids, antisense RNA, ribozymes, and RNAi oligonucleotides. In other embodiments, the compound is an antibody that specifically binds a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253. In yet other embodiments, the compound is a small molecule. In particular embodiments, the said compound increases the biological activity or expression of a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253. In particular embodiments, said compound decreases the biological activity or expression of a polypeptide having an amino acid sequence set forth in SEQ ID NOs: 49-97 and 179-253.

In another related embodiment, the present invention includes a plurality of polynucleotides consisting essentially of polynucleotides comprising a portion of a sequence set forth in SEQ ID NOs: 1-48 and 98-178 or a complement thereof. In one embodiment, the polynucleotides are single-stranded oligonucleotides. In another embodiment, the polynucleotides are labeled.

In a further embodiment, the present invention includes a plurality of antibodies consisting essentially of antibodies that specifically bind polypeptides having a sequence set forth in SEQ ID NOs: 49-97 and 179-253. In one embodiment, the antibodies are monoclonal antibodies. In one embodiment, the antibodies are labeled. In one embodiment, the polypeptides are secreted.

In various embodiments of the methods, kits, animals, cells, and pluralities of polynucleotides of the present invention, the polynucleotides and polypeptides comprise or consist of at least a portion of a TSC22D1 polynucleotide or polypeptide, including, *e.g.*, the TSC22D1 polynucleotides set forth in SEQ ID NOs: 111, 112, 5, and 6, and the TSC22D1 polypeptides set forth in SEQ ID NOs:

190, 191, 53, and 54. In related embodiments, the antibodies of the present invention specifically bind to a TSC22D1 polypeptide.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

Figures 1A and 1B are graphs depicting the liver iron content (1A) and spleen iron content (1B) in C57BL/6 mice loaded with the indicated amounts of iron for the indicated lengths of time.

Figure 2 shows four panels depicting Perls staining of liver sections obtained from two month old C57BL/6 mice loaded with the indicated amounts of iron. Iron (blue stain) is mainly localized in the hepatocytes of the periportal area.

Figure 3 depicts Tsc22d1 mRNA and TSC22D1 protein expression levels in iron-overloaded liver. Figure 3A is a graph showing Tsc22d1 mRNA level in the liver of mice fed by a diet supplemented with 0% or 3% carbonyl iron for 1, 2 and 4 months. White bar: 0% carbonyl-iron (n=4); black bar: 3% carbonyl-iron (n=5), *: $p \leq 0.05$ comparison to 0% at the same age, Mann and Whitney test. Figure 3B shows TSC22D1 protein expression in the liver of 0% and 3% carbonyl-iron mice at 2 months. Figure 3C is a graph showing Tsc22d1 mRNA expression level in liver of control-dextran and iron-dextran mice, 2 months after iron injection. White bar: Control-dextran liver (n=4); grey bar: Iron-dextran liver (n=5); *: $p \leq 0.05$ comparison to control dextran mice, Mann and Whitney test. Figure 4D shows TSC22D1 protein expression in the liver of control-dextran and iron-dextran mice at 2 months.

Figure 4 depicts iron concentration, expression of TSC22D1, and expression of hepcidin1 in the liver of mice fed an iron deficiency diet at 2, 4 and 30 days. Figure 4A is a graph showing Tsc22d1 mRNA level in liver of control and iron deficient mice at 2, 4 and 30 days. White bar: control mice (n=5 except for 30 days n=3); Black bar: iron deficient mice (n=5); *: $p \leq 0.05$ comparison to control mice at the same age, Mann and Whitney test. Figure 4B is a graph showing hepcidin1 mRNA expression level in the liver of control and iron-deficient mice at 2, 4 and 30 days. White bar: control mice (n=5 except for 30 days n=3); Black bar: iron deficient mice (n=5). Figure 4C is a graph showing liver iron concentration in iron-deficient mice at 2, 4 and 30 days. White Bar: control mice; Black bar: iron deficient mice. Figure 4D displays TSC22D1 protein expression in the liver of control and iron-deficient mice at 4 days.

Figure 5 depicts the expression of TSC22D1 and Hepcidin1 mRNA in the liver of anaemic mice. Figure 5A is a graph showing Tsc22d1 mRNA expression level in the liver of control and anemic mice. Figure 5B shows

TSC22D1 protein expression level in liver of control and anemic mice. White bar: control mice (n=5); Black bar: anemic mice (n=7).

Figure 6 displays the expression of TSC22D1 in hepatocytes and non parenchymal cells in the liver of iron-overloaded mice at 1 week and 2 months after the iron-dextran injection. Figure 6A shows Perls staining of liver of iron-dextran mice 1 week after the injection (X400) Gray arrows indicate non parenchymal cells overloaded. Figure 6B is a graph showing Tsc22d1 expression level in liver biopsy, non parenchymal cells and hepatocytes of control-dextran and iron-dextran mice, one week after the injection. White bar: control-dextran mice (n=4); Black bar: iron-dextran mice (n=4); *: $p \leq 0.05$ comparison to control-dextran non parenchymal cells, **: $p \leq 0.05$ comparison to control-dextran no parenchymal cells, Mann and Whitney test. Figure 6C shows Perls staining of liver of iron-dextran mice 2 months after the injection (X400) Gray arrows: non parenchymal cells, Black arrows: hepatocytes overloaded. Figure 6D is a graph showing Tsc22d1 expression level in liver biopsy, non parenchymal cells, and hepatocytes of control-dextran and iron-dextran mice, two months after the injection. White bar: control-dextran mice (n=3); Black bar: iron-dextran mice (n=3); *: $p \leq 0.05$ comparison to control-dextran mice, Mann and Whitney test.

Figure 7 demonstrates the effect of iron-citrate and deferroxamine in HuH7 cells on TFRC, TSC22D1 and HAMP expression level. White bar: HuH7 cells treated with citrate; Light grey bar: HuH7 cells treated with iron-citrate; Dark grey bar: HuH7 controls cells; Dark bar: HuH7 cells treated with Deferroxamine; *: $p \leq 0.05$ comparison to control mice, Mann and Whitney test. Figure 7A is a graph showing TFRC mRNA level in HUH7 cells treated with iron-citrate and deferroxamine during 48hours. Figure 7B is a graph showing TSC22D1 expression level in HUH7 cells treated with iron-citrate and deferroxamine during 48hours. Figure 7C is a graph showing HAMP expression level in HUH7 cells treated with iron-citrate and deferroxamine during 48hours.

Figure 8 demonstrates the effect of TSC22D1 inhibition in HuH7 cells on TFRC, HAMP, DMT1 mRNA expression and Ferritin protein. White bar: HuH7 treated with control siRNA, grey bar: HuH7 treated with TSC22D1 SiRNA n°1 (ref); black bar: HuH7 treated with TSC22D1 SiRNA n°2 (ref); *: $p \leq 0.05$ comparison to control mice, Mann and Whitney test. Figure 8A is a graph showing TSC22D1 mRNA expression level in HuH7 treated with SiRNA during 48h and 72h. Figure 8B is a graph showing TFRC mRNA expression level in HuH7 cells treated with SiRNA during 48h and 72h. Figure 8C is a graph showing C- HAMP mRNA expression level in HuH7 cells treated with SiRNA during 48h and 72h. Figure 8D

is a graph showing IRE-DMT1 mRNA expression level in HuH7 treated with SiRNA during 48h and 72h. Figure 8E displays Ferritin expression in HuH7 cells treated with TSC22D1 SiRNA for 48h and 72 h.

Figure 9 is a graph showing lactoferrin concentration in plasma in iron saturated genetic hemochromatosis, iron desaturated genetic hemochromatosis, cirrhosis and dysmetabolic hepatosiderosis. Control vs HGS $p<0.0001$; Control vs GHD $p<0,008$; Control vs GHD $p<0,0051$; HGS vs Cirrhosis $p<0.0136$.

DETAILED DESCRIPTION OF THE INVENTION

The identification of genes and polypeptides involved in iron homeostasis provides a better global understanding of the complex regulatory pathway of iron homeostasis and provides biomarkers for improved molecular testing for the early diagnosis of iron-related disorders. Such iron-related disorders may be a primary disease, or they may be secondary to another pathological condition, such as, *e.g.*, cirrhosis or neurological disorders. These genes and polypeptides may also be used as biomarkers of abnormal iron repartition within the body or within cells. In addition, genes involved in iron homeostasis and their encoded polypeptides are targets for therapeutic intervention in the treatment of iron-related disorders.

The present invention is based on the discovery of genes that are differentially expressed in an iron-related disorder. As described in the accompanying Examples, gene expression studies were performed in murine models of iron-related disorders, including the carbonyl iron overload model and the iron dextran model, using RNA-based microarray technology to identify panels of genes differentially regulated during iron loading. Accordingly, the present invention provides genes, and groups of genes, having expression profiles, mutations, or polymorphisms that are correlated with and predictive of iron-related disorders. According to the present invention, these genes and their encoded polypeptides, as well as compounds that modulate the expression or activity of these genes and their products, may be used in the diagnosis, treatment, and prevention of iron-related disorders.

As shown in Tables 2 and 3, it has been discovered according to the present inventors that the expression of a variety of genes is either increased or decreased in an animal model of iron overload. These genes are referred to herein as iron-related genes. Accordingly, any of these genes or groups of these genes may serve as biomarkers of iron-related disorders and as therapeutic

targets in the treatment of iron-related disorders. In certain embodiments, methods of the present invention are used to identify alterations in the expression or activity of one or more of these iron-related genes implicated in iron homeostasis, or an encoded polypeptide. Similarly, methods of treating or preventing iron-related disorders may involve modulating the activity or expression of one or more of these iron-related genes or their encoded polypeptides.

A variety of genes and their polypeptide products have been implicated in iron metabolism or homeostasis, and these genes also represent iron-related genes that may be used in methods, compositions, and kits of the present invention. In specific embodiments, one of the following iron-related genes or polypeptides is used as a biomarker or therapeutic target, alone or in combination with one or more other iron-related genes or polypeptides, including, but not limited to, any of those identified by the present invention or described herein.

Hepcidin, which is a small (25 amino acid active form) peptide hormone predominantly synthesized in the liver, plays important roles in iron homeostasis. Hepcidin serves to limit plasma iron by prevention of intestinal iron absorption and sequestration of iron in macrophages. Hepcidin has been directly implicated in iron overload syndromes, being inappropriately expressed for the degree of iron loading. In a severe form of human iron loading, called Juvenile Hemochromatosis, hepcidin is markedly deficient, resulting in excessive intestinal iron absorption.

Recently a liver-specific disruption of SMAD4 was shown to result in markedly decreased hepcidin expression and accumulation of iron in many organs. Furthermore, ectopic overexpression of SMAD4 activated the hepcidin promoter. Moreover, transcriptional activation of hepcidin is abrogated in SMAD4-deficient hepatocytes in response to iron overload, TGF-beta, BMP, or IL-6, overall demonstrating that TGF-beta/SMAD4 plays a key role in the regulation hepcidin.

TSC-22 (transforming growth factor-beta-stimulated clone 22; TSC22D1) belongs to a family of leucine zipper transcription factors. It is transcriptionally up-regulated by many different stimuli, including anti-cancer drugs and growth inhibitors. TSC-22 has been suggested to play a suppressive role in tumorigenesis. TSC-22 expression is regulated by several families of secreted signaling molecules in cultured cells. BMP-2/4 repress TSC-22 expression *in vivo*. Noggin, a BMP antagonist, promotes TSC-22 expression. Thus, TSC-22 is involved in BMP signaling.

Furthermore, hemojuvelin is a key protein involved in hepcidin regulation. Hemojuvelin is deficient in the majority of patients with Juvenile Hemochromatosis (JH) and this leads to the hepcidin deficiency and iron overload typical of JH patients. Recent studies have implicated hemojuvelin in the BMP pathway, clearly demonstrating that hemojuvelin is a BMP co-receptor and that hemojuvelin mutants associated with hemochromatosis have impaired BMP signaling ability. These studies further underscore the importance of TSC-22 in iron metabolism. Since TSC-22 has been clearly implicated in BMP signalling and the latter is also now well implicated via hemojuvelin in iron metabolism, this gene has significant potential both as a diagnostic marker for iron-related disease, but also as a therapeutic target for the treatment of these disorders.

Matrix metalloproteinases are a family of enzymes that are responsible for the degradation of extracellular matrix components. MMP-8, is a neutrophil collagenase enzyme. MMP-8 is closely related to the fibroblast collagenases (47% homology) but is encoded by a different gene. The enzyme exists as an inactive pro form with a molecular weight of approximately 85 kDa of which 35% is contributed by glycosylation. MMP-8 becomes activated by cleavage of the amino terminal pro-peptide.

It is well know that a complication of iron overload is fibrosis, which can develop into cirrhosis, with an increased risk of hepatocellular carcinoma. Since MMP8 is strongly regulated in the animal models described herein, showing inducibility with iron excess and attenuation with iron deficiency, it is possible that iron-induced fibrosis is modulated by MMP8. Thus, MMP8 levels have significant potential as a diagnostic biomarker predictive of the potential for the hepatic fibrotic state. This could also be of significance for other disorders of hepatic fibrosis, such as NASH, which has the potential to evolve into a cirrhotic/fibrotic state. In addition, given MMP8's critical biological role in modulating the extracellular matrix via collagen cleavage, it may also serve as a therapeutic target for the treatment of iron-induce fibrotic disorders, as well as disorders of fibrosis unrelated to iron metabolism.

Oxidative stress is associated with the development of numerous pathophysiopathological disorders. Elimination of free radicals (reactive oxygen species [ROS]) and their toxic products occurs through the oxidation of GSH to glutathione disulfide, subsequently regenerated by the glutathione reductase.

Vanin-1 is a member of a novel family of ectoenzymes including at least two proteins in mice (Vanin-1 and Vanin-3), three in humans (VNN1, VNN2, and VNN3), and *Drosophila* homologues. All Vanin molecules are pantetheinases

capable of specifically hydrolyzing pantetheine into pantothenic acid (vitamin B_5) and cysteamine, a sulfhydryl compound know for its antioxidant properties. The most important consequence observed in Vanin-$1^{-/-}$ mice is the lack of cysteamine in tissues where Vanin-1 expression is predominant. Vanin-$1^{-/-}$ mice are more resistant to paraquat poisoning and exposure to lethal doses of γ-irradiation. This protection is related to changes in the detoxifying potential of Vanin-$1^{-/-}$ tissues, characterized by elevated GSH stores. Importantly, intraperitoneal administration of cystamine (the disulfide form of cysteamine) abrogates the resistant phenotype of the mutant mice, suggesting that Vanin-1 regulates at least in part through cysteamine, the GSH-associated metabolism and modulates the adaptive tissue response to stress. Given Vanin's critical role in antioxidant metabolism, it is possible that therapeutic modulation of Vanin could have useful applications in the treatment of radiation induced damage or for the treatment of other pro-oxidant related disorders. Furthermore, vanin levels may serve as useful biomarkers predictive of the inflammatory and/or fibrotic state.

KIAA0101 is of keen interest as it harbors a novel motif with 65% homology to transferrin. This is a completely novel gene and because of its similarity to a key protein involved in iron metabolism, it too may well play a pivotal role in iron metabolism. It thus serves as an important diagnostic marker for both iron overload and deficiency, as well as other disorders of iron metabolism. In addition, it may have therapeutic utility as a target for iron-related diseases.

Lactoferrin is a protein previously identified to be involved in iron metabolism. Lactoferrin is a glycoprotein that belongs to the iron transporter or transferrin family. Lactoferrin contains 703 amino acids and has a molecular weight of 80 kilodaltons. In addition to its presence in milk, it is also found in exocrine secretions of mammals and is released from neutrophil granules during inflammation.

Lactoferrin is considered a multifunctional protein. It appears to play several biological roles. Owing to its iron-binding properties, lactoferrin is thought to play a role in iron uptake by the intestinal mucosa of the suckling neonate serving as a source of iron for breast-fed infants. It also appears to have antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and immunomodulatory activities.

Lactoferrin is well regulated in the animal models described herein, showing inducibility with iron excess and attenuation with iron deficiency. It is, therefore, believed to be a biomarker for body iron status. In addition, given its

diverse biological roles, it may also serve as a therapeutic target for iron-metabolism disorders.

Definitions:

"Iron-related genes," "iron-related polynucleotides," "iron-related nucleic acids," and "iron related polypeptides," as used herein, refer to genes, polynucleotides, and polypeptides involved in iron regulation, iron homeostasis, or iron metabolism, as well as genes, polynucleotides, and polypeptides whose expression is regulated by iron, including those identified as having altered expression levels as compared to normal in Tables 2-4. Sequence identifiers for polynucleotides and polypeptides listed in these tables are provided in Table 1. It is understood that certain of these polynucleotide sequence may include non-coding sequence, and it is understood that in certain embodiments of the present invention, only the coding regions are utilized. Accordingly, methods and composition of the present invention may be directed, in various embodiments, to any polynucleotide that encodes a polypeptide encoded by a polynucleotide sequence set forth herein.

Table 1. Sequence identifiers for iron-related cDNAs and polypeptides

Mouse symbol	Mouse mRNA Refseq and SEQ ID NO	Mouse Protein Refseq and SEQ ID NO	Human symbol	Human mRNA Refseq and SEQ ID NO	Human Protein Refseq and SEQ ID NO
Apoa4	NM_007468 SEQ ID NO:1	NP_031494 SEQ ID NO:49	APOA4	NM_000482 SEQ ID NO:98	NP_000473 SEQ ID NO:179
Onecut1	NM_008262 SEQ ID NO:2	NP_032288 SEQ ID NO:50	ONECUT1	NM_004498 SEQ ID NO:99	NP_004489 SEQ ID NO:180
Id2	NM_010496 SEQ ID NO:3	NP_034626 SEQ ID NO:51	ID2	NM_002166 SEQ ID NO:100	NP_002157 SEQ ID NO:181
Mt1	NM_013602 SEQ ID NO:4	NP_038630 SEQ ID NO:52	MT1A MT1B MT1E MT1F MT1G MT1H MT1JP MT1L MT1M MT1X	NM_005946 SEQ ID NO:101 NM_005947 SEQ ID NO:102 NM_175617 SEQ ID NO:103 NM_005949 SEQ ID NO:104 NM_005950 SEQ ID NO:105 NM_005951 SEQ ID NO:106 NM_175622 SEQ ID NO:107 NM_002450 SEQ ID NO:108 NM_176870 SEQ ID NO:109 NM_005952 SEQ ID NO:110	NP_005937 SEQ ID NO:182 NP_005938 SEQ ID NO:183 NP_783316 SEQ ID NO:184 NP_005940 SEQ ID NO:185 NP_005941 SEQ ID NO:186 NP_005942 SEQ ID NO:187 NP_789846 SEQ ID NO:188 NP_005943 SEQ ID NO:189
Tsc22d1	NM_009366 SEQ ID NO:5 NM_207652 SEQ ID NO:6	NP_033392 SEQ ID NO:53 NP_997535 SEQ ID NO:54	TSC22D1	NM_183422 SEQ ID NO:111 NM_006022 SEQ ID NO:112	NP_904358 SEQ ID NO:190 NP_006013 SEQ ID NO:191
Dopey2	NM_026700 SEQ ID NO:7 NM_027293 SEQ ID NO:8	NP_080976 SEQ ID NO:55 NP_081569 SEQ ID NO:56	DOPEY2	NM_005128 SEQ ID NO:113	NP_005119 SEQ ID NO:192
Vegfb	NM_011697	NP_035827	VEGFB	NM_003377	NP_003368

Mouse symbol	Mouse mRNA Refseq and SEQ ID NO	Mouse Protein Refseq and SEQ ID NO	Human symbol	Human mRNA Refseq and SEQ ID NO	Human Protein Refseq and SEQ ID NO
	SEQ ID NO:9	SEQ ID NO:57		SEQ ID NO:114	SEQ ID NO:193
Ugt3a2	NM_144845 SEQ ID NO:10 XM_001052104 SEQ ID NO:11	NP_659094 SEQ ID NO:58 XP_001052104 SEQ ID NO:59	UGT3A2	NM_174914 SEQ ID NO:115	NP_777574 SEQ ID NO:194
Vnn1	NM_011704 SEQ ID NO:12	NP_035834 SEQ ID NO:60	VNN1	NM_004666 SEQ ID NO:116	NP_004657 SEQ ID NO:195
Col27a1	NM_025685 SEQ ID NO:13	NP_079961 SEQ ID NO:61	COL27A1	NM_032888 SEQ ID NO:117	NP_116277 SEQ ID NO:196
Pparg	NM_011146 SEQ ID NO:14	NP_035276 SEQ ID NO:62	PPARG	NM_005037 SEQ ID NO:118 NM_015869 SEQ ID NO:119 NM_138711 SEQ ID NO:120 NM_138712 SEQ ID NO:121	NP_005028 SEQ ID NO:197 NP_056953 SEQ ID NO:198 NP_619725 SEQ ID NO:199 NP_619726 SEQ ID NO:200
Ugt3a1	NM_207216 SEQ ID NO:15	NP_997099 SEQ ID NO:63	UGT3A1	NM_152404 SEQ ID NO:122	NP_689617 SEQ ID NO:201
Hsd17b6	NM_013786 SEQ ID NO:16	NP_038814 SEQ ID NO:64	HSD17B6	NM_003725 SEQ ID NO:123	NP_003716 SEQ ID NO:202
Mmp8	NM_008611 SEQ ID NO:17	NP_032637 SEQ ID NO:65	MMP8	NM_002424 SEQ ID NO:124	NP_002415 SEQ ID NO:203
Ltf	NM_008522 SEQ ID NO:18	NP_032548 SEQ ID NO:66	LTF	NM_002343 SEQ ID NO:125	NP_002334 SEQ ID NO:204
Dyrk3	NM_145508 SEQ ID NO:19	NP_663483 SEQ ID NO:67	DYRK3	NM_001004023 SEQ ID NO:126 NM_003582 SEQ ID NO:127	NP_001004023 SEQ ID NO:205 NP_003573 SEQ ID NO:206
2810417H13Rik	NM_026515 SEQ ID NO:20	NP_080791 SEQ ID NO:68	KIAA0101	NM_001029989 SEQ ID NO:128 NM_014736 SEQ ID NO:129	NP_001025160 SEQ ID NO:207 NP_055551 SEQ ID NO:208
Car2	NM_009801 SEQ ID NO:21	NP_033931 SEQ ID NO:69	CA2	NM_000067 SEQ ID NO:130	NP_000058 SEQ ID NO:209
Chek1	NM_007691 SEQ ID NO:22	NP_031717 SEQ ID NO:70	CHEK1	NM_001274 SEQ ID NO:131	NP_001265 SEQ ID NO:210
Chi3l3	NM_009892 SEQ ID NO:23	NP_034022 SEQ ID NO:71			
Hsp90aa1	NM_010480 SEQ ID NO:24	NP_034610 SEQ ID NO:72	HSP90AA1	NM_001017963 SEQ ID NO:132 NM_005348 SEQ ID NO:133	NP_001017963 SEQ ID NO:211 NP_005339 SEQ ID NO:212
Spna1	NM_011465 SEQ ID NO:25	NP_035595 SEQ ID NO:73	SPTA1	NM_003126 SEQ ID NO:134	NP_003117 SEQ ID NO:213
Aurka	NM_011497 SEQ ID NO:26	NP_035627 SEQ ID NO:74	AURKA	NM_003600 SEQ ID NO:135 NM_198433 SEQ ID NO:136 NM_198434 SEQ ID NO:137 NM_198435 SEQ ID NO:138 NM_198436 SEQ ID NO:139 NM_198437 SEQ ID NO:140	NP_003591 SEQ ID NO:214 NP_950835 SEQ ID NO:215
D6Wsu116e	NM_026585 SEQ ID NO:27	NP_080861 SEQ ID NO:75	FAM21A (RP11_56A21.1) FAM21B FAM21C	NM_001005751 SEQ ID NO:141 XM_290482 SEQ ID NO:142 NM_015262 SEQ ID NO:143	NP_001005751 SEQ ID NO:216 XP_290482 SEQ ID NO:217 NP_056077 SEQ ID NO:218
Hfe2	NM_027126 SEQ ID NO:28	NP_081402 SEQ ID NO:76 Q7TQ32 SEQ ID NO:77	HFE2	NM_145277 SEQ ID NO:144 NM_202004 SEQ ID NO:145 NM_213652 SEQ ID NO:146	NP_660320 SEQ ID NO:219 NP_973733 SEQ ID NO:220 NP_998817 SEQ ID NO:221

Mouse symbol	Mouse mRNA Refseq and SEQ ID NO	Mouse Protein Refseq and SEQ ID NO	Human symbol	Human mRNA Refseq and SEQ ID NO	Human Protein Refseq and SEQ ID NO
				NM_213653 SEQ ID NO:147	NP_998818 SEQ ID NO:222
Hamp1	NM_032541 SEQ ID NO:29	NP_115930 SEQ ID NO:78	HAMP	NM_021175 SEQ ID NO:148	NP_066998 SEQ ID NO:223
LOC383891	XM_357312 SEQ ID NO:30	XP_357312 SEQ ID NO:79	FTL	NM_000146 SEQ ID NO:149	NP_000137 SEQ ID NO:224
Fth1	NM_010239 SEQ ID NO:31	NP_034369 SEQ ID NO:80	FTH1	NM_002032 SEQ ID NO:150	NP_002023 SEQ ID NO:225
Tfrc	NM_011638 SEQ ID NO:32	NP_035768 SEQ ID NO:81	TFRC	NM_003234 SEQ ID NO:151	NP_003225 SEQ ID NO:226
Trf	NM_133977 SEQ ID NO:33	NP_598738 SEQ ID NO:82	TF	NM_001063 SEQ ID NO:152	NP_001054 SEQ ID NO:227
Cat	NM_009804 SEQ ID NO:34	NP_033934 SEQ ID NO:83	CAT	NM_001752 SEQ ID NO:153	NP_001743 SEQ ID NO:228
Cp	NM_007752 SEQ ID NO:35	NP_031778 SEQ ID NO:84	CP	NM_000096 SEQ ID NO:154	NP_000087 SEQ ID NO:229
Hp	NM_017370 SEQ ID NO:36	NP_059066 SEQ ID NO:85	HP	NM_005143 SEQ ID NO:155	NP_005134 SEQ ID NO:230
Hpxn	NM_017371 SEQ ID NO:37	NP_059067 SEQ ID NO:86	HPX	NM_000613 SEQ ID NO:156	NP_000604 SEQ ID NO:231
Cd163	NM_053094 SEQ ID NO:38	NP_444324 SEQ ID NO:87	CD163	NM_203416 SEQ ID NO:157 NM_004244 SEQ ID NO:158	NP_981961 SEQ ID NO:232 NP_004235 SEQ ID NO:233
Epo	NM_007942 SEQ ID NO:39	NP_031968 SEQ ID NO:88	EPO	NM_000799 SEQ ID NO:159	NP_000790 SEQ ID NO:234
Il6	NM_031168 SEQ ID NO:40	NP_112445 SEQ ID NO:89	IL6	NM_000600 SEQ ID NO:160	NP_000591 SEQ ID NO:235
Tnf	NM_013693 SEQ ID NO:41	NP_038721 SEQ ID NO:90	TNF	NM_000594 SEQ ID NO:161	NP_000585 SEQ ID NO:236
Hmox1	NM_010442 SEQ ID NO:42	NP_034572 SEQ ID NO:91	HMOX1	NM_002133 SEQ ID NO:162	NP_002124 SEQ ID NO:237
Bmp2	NM_007553 SEQ ID NO:43	NP_031579 SEQ ID NO:92	BMP2	NM_001200 SEQ ID NO:163	NP_001191 SEQ ID NO:238
Slc11a2	NM_008732 SEQ ID NO:44	NP_032758 SEQ ID NO:93	SLC11A2	NM_000617 SEQ ID NO:164	NP_000608 SEQ ID NO:239
Cybrd1	NM_028593 SEQ ID NO:45	NP_082869 SEQ ID NO:94	CYBRD1	NM_024843 SEQ ID NO:165	NP_079119 SEQ ID NO:240
Slc40a1	NM_016917 SEQ ID NO:46	NP_058613 SEQ ID NO:95	SLC40A1	NM_014585 SEQ ID NO:166	NP_055400 SEQ ID NO:241
Hfe	NM_010424 SEQ ID NO:47	NP_034554 SEQ ID NO:96	HFE	NM_139005 SEQ ID NO:167 NM_139002 SEQ ID NO:168 NM_000410 SEQ ID NO:169 NM_139004 SEQ ID NO:170 NM_139003 SEQ ID NO:171 NM_139009 SEQ ID NO:172 NM_139007 SEQ ID NO:173 NM_139008 SEQ ID NO:174 NM_139010 SEQ ID NO:175 NM_139011 SEQ ID NO:176 NM_139006 SEQ ID NO:177	NP_620574 SEQ ID NO:242 NP_620571 SEQ ID NO:243 NP_000401 SEQ ID NO:244 NP_620573 SEQ ID NO:245 NP_620572 SEQ ID NO:246 NP_620578 SEQ ID NO:247 NP_620576 SEQ ID NO:248 NP_620577 SEQ ID NO:249 NP_620579 SEQ ID NO:250 NP_620580 SEQ ID NO:251 NP_620575 SEQ ID NO:252
Trfr2	NM_015799 SEQ ID NO:48	NP_056614 SEQ ID NO:97	TFR2	NM_003227 SEQ ID NO:178	NP_003218 SEQ ID NO:253

"Differentially expressed" means a change in the level of expression of a genetic sequence, *e.g.*, a gene or RNA, or a protein, under one set of conditions as compared to another. The genetic sequence or protein can be either upregulated or downregulated as compared to the reference. In certain embodiments, the change represents an increase of at least two-fold, at least three-fold, at least five-fold or at least ten-fold. In other embodiments, the change represent a decrease to less than 90%, less than 75%, less than 50%, less than 25%, or less than 10%. When using a panel incorporating multiple iron-related genes, it is contemplated that each gene can be either upregulated or downregulated separately from the other as compared to their respective reference.

"Disease" and "disorder" refers to any abnormal biological state that is detrimental to a cell, tissue, organ, or organism, and includes, but is not limited to an interruption, cessation, or abnormal function. With respect to the present invention, any disruption to iron homeostasis, such as iron overload or iron deficiency or any associated conditions is considered to be a disease or disorder. Further, diseases and disorders can be categorized into different levels of disease or disorder, which is an arbitrary measure that reflects the progression of a disease or disorder and often becomes increasingly more severe.

"Expression profile" refers to the measurement of the relative abundance of a plurality of cellular constituents. Such measurements may include, RNA or protein abundance or activity levels. For example, the expression profiles can be a measurement of the transcriptional or the translation state. The comparison of gene expression profiles from an experimental sample and a reference sample to identify genes that are differently expressed between two biological states has become a powerful diagnostic and prognostic tool.

A. Methods of Detecting Iron-Related Disorders

The present invention provides methods of diagnosing an iron-related disorders or detecting a risk for having or developing an iron-related disorder in a patient. In addition, these methods may be used to diagnosis or detect a risk of abnormal iron repartition within the body, or within a cell, *e.g.*, by detecting reduced or increased expression or presence of an iron-related gene or polypeptide within one or more tissues or organs, or cellular compartments or organelles. The methods may be readily adapted to monitor the progression or regression of an iron-related disease or disorder, or the efficacy of treatment.

According to the present invention, iron-related disorders are associated with alterations in the expression or activity of one or more iron-related genes, including those listed in Tables 2-4. Such alteration may occur within an entire animal, within one or more organs, tissues, or cells types, or even within specific cellular compartments or organelles. Therefore, expression, biological activity, and mutational analysis of these genes and their polynucleotide and polypeptide products can each serve as a diagnostic tool for iron-related disorders. These iron-related disorders may be the primary cause of disease or pathology, or they may result secondary to another pathological condition, such as, *e.g.*, cirrhosis or neurological disorders. In addition, determination of iron-related gene sequences can be used to subtype individuals or families to determine their predisposition for developing a particular iron-related disorder. These methods may be readily adapted to monitor the progression of an iron-related disorder or assess a patient's response to treatment of an iron-related disorder.

In particular embodiments, methods of the present invention may be used to diagnose specific classes of iron-related disorders, as well as particular disorders, *e.g.*, based upon the expression of genes and polypeptides having altered expression patterns associated with particular iron-related disorders. For example, iron overload disorders may be identified based upon the overexpression or underexpression of genes or encoded polypeptides identified in Tables 2 or 3 as being overexpressed or underexpressed, respectively, in iron overload disease models. Similarly, disorders associated with iron deficiency, such as anemia, may be identified based upon the underexpression or overexpression of genes or encoded polypeptides identified in Tables 2 or 3 as being overexpressed or underexpressed, respectively, in iron overload disease models. In addition, disorders associated with iron deficiency, may be identified based upon the overexpression or underexpression of genes or encoded polypeptides identified in Table 3 as being overexpressed or underexpressed, respectively, in an iron deficiency disease model, such as anemia. In certain embodiment, the expression of a panel of two of more of these genes or encoded polypeptides is examined, and the type of iron-related disorder is determined by correlating the expression patterns of the panel of genes or encoded polypeptides in a patient with the expression patterns in one or more patients diagnosed with a particular iron-related disorder.

In particular embodiments, a disease or disorder associated with iron overload is diagnosed based upon a reduced level of expression or activity of a Tsc22d1 polynucleotide or TSC22D1 polypeptide in a biological sample obtained

from a subject. In particular embodiments, a disease or disorder associated with iron deficiency, such as AI, is diagnosed based upon an increased level of expression or activity of a Tsc22d1 polynucleotide or TSC22D1 polypeptide in a biological sample obtained from a subject. In one embodiment, the biological sample is a tissue sample, *e.g.*, a tissue sample obtained from the subject's liver.

Patients may be any animal, including both humans and non-human animals. In certain embodiments, patients are mammals. Patients may be male or female, and may be adult or juvenile. In particular embodiments, patients may be diagnosed with an iron-related disorder or considered at risk of having or developing an iron-related disorder.

The methods of the present invention may be used to diagnosis or treat any iron-related disorder, including, but not limited to, iron deficiency, AI, and iron overload. Other examples of disorders caused by too much iron include cirrhosis, liver cancer, osteoarthritis, osteopenia, osteomalacia, diabetes, irregular heart beat, heart attack, hypothyroidism, infertility, impotence, depression, hypogonadism, and bronze or ashen gray skin miscoloration. Examples of other iron-related disorders that may be diagnosed and treated according to the present invention include, *e.g.*, hemochromatosis, juvenile hemochromatosis, acquired iron overload, sickle cell anemia, thalassemia, African siderosis, porphyria cutaena tarda, iron deficiency anemia, Friedreich Ataxia, ferroportin disease, hyperferritinemia, atransferrinemia, and sideroblastic anemia. Iron-related disorders further include, *e.g.*, heart failure, haemolytic anaemia, and neurological disorders.

In certain embodiments, the methods of the present invention comprise measuring the level of activity or expression of one or more iron-related polynucleotides or polypeptides in a biological sample obtained from a patient, and then comparing the level measured to a control value or to a level measured in a control subject that does not have the iron-related disorder. If the level in the patient is significantly higher or lower than the control level, then the patient may be considered to have or be at risk of having the iron-related disorder. In certain embodiments, the patient is considered to have the iron-related disorder if the level in the patient is at least two-fold, three-fold, or five-fold different than the level in the control. In one embodiment, a patient is diagnosed with an iron-related disorder if the level of expression or activity of an iron-related polynucleotide or polypeptide is significantly lower than a control value. In certain embodiments, the level of expression or activity in the patient is less than 75%, less than 50%, less than 25%, or less than 10% the level measured in the control.

Methods of determining levels or amounts of specific polynucleotides and polypeptides in a biological sample are known in the art. One exemplary method for measuring levels of an iron-related polynucleotide in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of binding an iron-related polynucleotide (*e.g.*, mRNA, genomic DNA), and then detecting an amount of bound agent. In certain embodiments, the agent is a labeled nucleic acid probe capable of hybridizing to an iron-related polynucleotide. The nucleic acid probe may be, for example, a full-length iron-related polynucleotide, such as those set forth in SEQ ID NOs:1-48 and 98-178, or a portion or complement thereof, such as an oligonucleotide of at least 12, 15, 30, 50, 100, 250 or 500 nucleotides in length that specifically hybridizes under stringent conditions to an iron-related polynucleotide, *e.g.*, mRNA or genomic DNA. Suitable stringent conditions for testing the hybridization of a polynucleotide of this invention with other polynucleotides include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 65° C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS.

Another method for detecting levels of an iron-related protein in a biological sample involves obtaining a biological sample from a patient and contacting the biological sample with an antibody that is capable of specifically binding an iron-related polypeptide, and then detecting the amount of bound antibody. In particular embodiments, the antibody is detectably labeled. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or F(ab')2) can also be used.

The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The labels may be incorporated by any of a number of means well known to those of skill in the art. Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate,

magnetic beads (*e.g.*, Dynabeads™), fluorescent dyes (*e.g.*, fluorescein, texas red, rhodamine, green fluorescent protein, yellow fluorescent protein and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex, etc.) beads. Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label.

The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect iron-related polynucleotides and polypeptides in a biological sample *in vitro* as well as *in vivo*. A preferred biological sample is a serum sample isolated by conventional means from a subject. In particular embodiments, methods of the present invention are practiced using one or more compounds that specifically bind secreted iron-related polypeptides, such as those identified in Table 4, to determine the amount of the secreted polypeptides present in a blood or serum sample from a patient, wherein an alteration in the amount in the serum sample from the patient as compared to a serum sample from a normal control or control value, indicates that the patient has an iron-related disorder.

Examples of *in vitro* techniques for detection and quantification of mRNA include polymerase chain reactions (PCR), including reverse transcriptase-PCR (RT-PCR) and quantitative PCR, as well as Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection and quantification of protein include enzyme linked immunosorbent assays (ELISAs), fluorescence activated cell sorting (FACS) analysis, Western blots, immunoprecipitations and immunofluorescence. *In vitro* techniques for detection of genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for detection of a protein include introducing into a patient a labeled antibody that specifically binds an iron-related polypeptide. For example, the antibody can be labeled with a radioactive marker, allowing its presence and location in a subject to be detected by standard imaging techniques.

The present invention further provides a method of establishing an iron-related gene expression profile for a subject or patient, comprising measuring the level of expression of one or more iron-related genes (*e.g.*, mRNA expression levels) in a biological sample obtained from the subject or patient. The measured levels constitute a gene expression profile.

In certain embodiments, the method of the present invention includes a step of comparing the level of expression of the one or more iron-related genes to a control in order to determine whether there is any observed change or difference in expression of each gene in the patient as compared to the control. As discussed above, the present inventors have identified the expression profile of multiple genes that are differentially regulated in iron-related disorders as compared to a "normal" control (*i.e.*, a patient that does not have an iron-related disorder), including the manner in which the genes are regulated. Therefore, one can determine whether a patient has a gene expression profile that is statistically substantially similar to the profile of gene expression of a patient with an iron-related disorder or whether a profile of gene expression in the patient is statistically more similar to the negative or normal, non-disease control.

According to the present invention, an expression profile is substantially similar to a given profile of expression established for a group if the expression profile of the gene or genes detected (including the identity of the gene, the manner in which expression is regulated, and/or the level of expression of the gene) is similar enough to the expected result so as to be statistically significant (*i.e.*, with at least a 95% confidence level, or $p<0.05$, and more preferably, with a confidence level of $p<0.01$, and even more preferably, with a confidence level of $p<0.005$, and even more preferably, with a confidence level of $p<0.001$). Software programs are available in the art that are capable of analyzing the expression of multiple genes and determining whether differences from a control are significant or not significant. In addition, statistical analysis methods are known in the art that are preferably used to analyze the expression data generated for patient samples (*e.g.*, independent and "leave-one-out" cross-validation and/or permutation testing).

The methods of the present invention may be performed by analyzing the expression or activity of one or more than one, *i.e.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, etc., iron-related polynucleotide or polypeptide. For example, in certain embodiments, a panel comprising two or more iron-related polynucleotides or polypeptides is examined. In particular embodiments, the iron-related polypeptides are secreted proteins.

In various embodiments, methods of detecting or quantitating iron-related polynucleotides and polypeptides may be performed in solution or with a solid support. For example, in certain embodiments, agents that specifically bind to an iron-related polynucleotide or polypeptide are attached to a solid support, *e.g.*, covalently or non-covalently, either before or after being contacted with a biological sample potentially containing the iron-related polynucleotide or polypeptide to which they bind. The attached agent and bound iron-related polynucleotides or polypeptides may then be washed to remove unbound biological sample, thereby facilitating detection of bound polynucleotides and polypeptides. In certain embodiments, solid supports are beads, microplates (*e.g.*, glass slides), or blotting membranes. Oligonucleotide or antibody arrays, including high density microarrays, may also be used in the present invention.

In one embodiment, the present invention includes methods of determining the level of expression of two or more iron-related polynucleotides, comprising contacting a solid support having two or more oligonucleotides attached thereto at discrete locations, wherein said oligonucleotides correspond to a fragment of an iron-related polynucleotide sequence set forth in SEQ ID NOs:1-48 and 98-178 or a complement thereof, with polynucleotides obtained from a biological sample obtained from a patient, and then determining the amount of polynucleotide bound to each oligonucleotide. These amounts are then compared to normal control values to determine if they are higher or lower than normal, thereby indicating that the patient has an iron-related disorder. In certain embodiments, polynucleotides in the biological sample are labeled to facilitate detection. In particular embodiments, the oligonucleotides are 20-80-mer oligonucleotides. In other embodiments, the method is performed using peptide nucleic acid (PNA) probes. In particular embodiments, the polynucleotides are mRNA.

Nucleic acid arrays are particularly useful for detecting the expression of the polynucleotides of the present invention. The production and application of high-density arrays in gene expression monitoring have been disclosed previously in, for example, PCT Publication No. WO 97/10365; PCT Publication No. WO 92/10588; U.S. Pat. No. 6,040,138; U.S. Pat. No. 5,445,934; or PCT Publication No. WO 95/35505. Also, for examples of arrays, see Hacia *et al.* (1996) Nature Genetics 14:441-447; Lockhart *et al.* (1996) Nature Biotechnol. 14:1675-1680; and De Risi *et al.* (1996) Nature Genetics 14:457-460. In general, in an array, an oligonucleotide, a cDNA, or genomic DNA, that is a portion of an iron-related polynucleotide, or a complement thereof, occupies a known location on a

substrate. A nucleic acid target sample is hybridized with an array of such oligonucleotides, and then the amount of target nucleic acids hybridized to each probe in the array is quantified. One preferred quantifying method is to use confocal microscope and fluorescent labels. The Affymetrix GeneChip™ Array system (Affymetrix, Santa Clara, CA) and the Atlas™ Human cDNA Expression Array system are particularly suitable for quantifying the hybridization; however, it will be apparent to those of skill in the art that any similar systems or other effectively equivalent detection methods can also be used. In one embodiment, knowledge of the genes described herein is used to design novel arrays of iron-related polynucleotides, cDNAs or genomic DNAs for screening methods described herein. Such novel pluralities of polynucleotides are contemplated to be a part of the present invention and are described in detail below. An array may further comprise one or more control oligonucleotides that bind a polynucleotide expressed by a gene that is not regulated by iron levels.

Suitable nucleic acid samples for screening on an array contain transcripts of interest or nucleic acids derived from the transcripts of interest (*i.e.*, transcripts derived from the iron-related genes described herein). As used herein, a nucleic acid derived from a transcript refers to a nucleic acid for whose synthesis the mRNA transcript or a subsequence thereof has ultimately served as a template. Thus, a cDNA reverse transcribed from a transcript, an RNA transcribed from that cDNA, a DNA amplified from the cDNA, an RNA transcribed from the amplified DNA, *etc.*, are all derived from the transcript and detection of such derived products is indicative of the presence and/or abundance of the original transcript in a sample. Thus, suitable samples include, but are not limited to, transcripts of the gene or genes, cDNA reverse transcribed from the transcript, cRNA transcribed from the cDNA, DNA amplified from the genes, RNA transcribed from amplified DNA, and the like. Preferably, such a sample is a total RNA isolated from a biological sample.

The present invention further provides methods of detecting an iron-related disorder or risk thereof in a patient comprising measuring the level of one or more biological activities of a polypeptide encoded by an iron-related gene. Biological activities known to be associated with an iron-related polypeptide may be assayed using methods known in the art, depending upon the particular activity being examined. Assays that may be used include cell-based assays such as: cytokine secretion assays, or intracellular signal transduction assays that determine, for example, protein or lipid phosphorylation, mediator release or

intracellular Ca^{++} mobilization. In one embodiment, the polypeptide is a TSC22D1 polypeptide.

The present invention further provides methods of diagnosing an iron-related disorder or risk thereof in a patient based upon detecting a mutation or polymorphism in an iron-related gene, including any described herein. A gene encoding an iron-related polypeptide may have a mutation or polymorphism that is a causative or risk factor of an iron-related disorder. Methods of identifying mutations and polymorphisms are well-known in the art and include, *e.g.*, PCR, RT-PCR, northern blot (*e.g.*, using clones encompassing discrete regions of cDNA), Southern blot, polymorphic specific probes, sequencing analysis, hybridization assays, restriction endonuclease analysis, and exon-specific amplification. In one embodiment, the gene is a Tsc22d1 gene.

Accordingly, methods of the invention can be used to detect genetic alterations in an iron-related gene, thereby determining if a patient with the altered gene is at risk for an iron-related disorder. In preferred embodiments, the methods include detecting, in a biological sample from the patient, the presence or absence of a genetic alteration characterized by an alteration affecting the integrity of a gene encoding an iron-related polypeptide, or aberrant expression of the iron-related gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of: 1) a deletion of one or more nucleotides from the gene; 2) an addition of one or more nucleotides to the gene; 3) a substitution of one or more nucleotides of the gene, 4) a chromosomal rearrangement of the gene; 5) an alteration in the level of a messenger RNA transcript of the gene, 6) aberrant modification of the gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of the gene, 8) a non-wild type level of a protein, 9) allelic loss of the gene, and 10) inappropriate post-translational modification of a protein. There are a large number of assays known in the art that can be used for detecting alterations in an iron-related gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject, *i.e.*, patient.

In certain embodiments, detection of a mutation or polymorphism involves the use of a probe/primer in a polymerase chain reaction (PCR) such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (*see, e.g.*, Landegran *et al.* (1988) Science 241:1077-1080; and Nakazawa *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:360-364), the latter of which can be particularly useful for detecting point mutations (see Abravaya *et al.* (1995) Nucleic

Acids Res. 23:675-682). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (*e.g.*, genomic DNA, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers that specifically hybridize to the iron-related nucleic acid under conditions such that hybridization and amplification of the iron-related nucleic acid (if present) occurs, and detecting the presence or absence, or an amount of, an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include, *e.g.*, self sustained sequence replication (Guatelli, J. C. *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. *et al.*, (1989) Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi, P. M. *et al.* (1988) Bio-Technology 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art.

In an alternative embodiment, mutations in an iron-related gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. In other embodiments, genetic mutations in iron-related genes can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin, M. T. *et al.* (1996) Human Mutation 7: 244-255; Kozal, M. J. *et al.* (1996) Nature Medicine 2: 753-759). For example, genetic mutations in iron-related genes can be identified using two dimensional arrays containing light-generated DNA probes as described in Cronin, M. T. *et al.* (1996) Human Mutation 7: 244-255.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the iron-related gene or cDNA and detect mutations by comparing the sequence of the sample iron-related gene sequence with the corresponding wild-type (control) sequence. Other methods for detecting mutations include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) Science 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes of formed by hybridizing (labeled) RNA or DNA containing the wild-type sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent that cleaves single-stranded regions of the duplex such as which will

exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with SI nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. For examples see, Cotton *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:4397; and Saleeba *et al.* (1992) Methods Enzymol. 217:286-295. The control DNA or RNA can be labeled for detection.

In other embodiments, alterations in electrophoretic mobility are used to identify mutations iron-related genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) Proc. Natl. Acad. Sci. USA: 86:2766, see also Cotton (1993) Mutat. Res. 285:125-144; and Hayashi (1992) Genet. Anal. Tech. Appl. 9:73-79).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) Nature 324:163); Saiki *et al.* (1989) Proc. Natl. Acad. Sci. USA 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA. Alternatively, allele specific amplification technology, which depends on selective PCR amplification, may be used.

The methods described herein can be used to determine whether a patient should be administered a therapeutic agent to treat an iron-related disorder. Thus, the present invention provides methods for determining whether a patient can be effectively treated with an agent for a disorder associated with aberrant iron-related gene expression or activity, in which a biological sample is obtained from a patient and a level of iron-related protein or nucleic acid expression or activity is detected (*e.g.*, wherein the level of iron-related protein or nucleic acid expression or activity is diagnostic for a subject that can be administered the agent to treat an iron-related disorder). In certain embodiments, the methods are used to distinguish whether a patient diagnosed with anemia

suffers from AI or true iron deficiency, thereby indicating what therapeutic regime should be followed. AI is an iron deficient state characterized by low transferrin saturation and low serum iron, which often can be associated with hyperferritenemia. The state is related to inflammation as demonstrated by C-reactive protein (CRP) increases. The mechanism involves iron sequestration within macrophages and impaired erythropoiesis. Iron deficiency is related to iron losses or low iron absorption and is characterized by a decrease in transferrin saturation, a decrease in serum iron, and reduced plasma ferritin. The anemia in iron deficiency is related to a decrease in global iron storage.

Methods of the present invention may be adapted for monitoring the progression of an iron-related disorder in a patient. For example, in one embodiment, the relative level of expression or activity of one or more iron-related polynucleotides or polypeptides, as compared to a normal control level, is determined at two different time points during the course of a disorder. If the relative level is closer to a normal control level at the later time point, it indicates that the disorder may be lessening or entering remission. If the relative level is further from a normal control level at the later time point, it indicates that the disorder is progressing or becoming more severe. In particular embodiments, the polynucleotide or polypeptide is a TSC22D1 polynucleotide or polypeptide.

Methods of the present invention may be adapted for monitoring a patient's response to a therapeutic agent or treatment. For example, in one embodiment, the relative level of expression or activity of one or more iron-related polynucleotides or polypeptides, as compared to a normal control level, is determined at two different time points, *e.g.*, prior to treatment and following treatment. If the relative level is closer to a normal control level following treatment or administration of a therapeutic agent, it indicates that the treatment or agent is efficacious. If the relative level is further from a normal control level following treatment or administration of a therapeutic agent, it indicates that the treatment or agent is not efficacious. The two points may occur at any point before, during, or after treatment, or any combination thereof. In particular embodiments, the polynucleotide or polypeptide is a TSC22D1 polynucleotide or polypeptide.

Diagnostic and prognostic methods of the present invention may be practiced by measuring the expression or activity of any combination of iron-related genes or polypeptides, including combinations comprising iron-related polypeptides identified in Tables 2 and 3 as having altered expression in iron-related disorders in combination with genes or polypeptides previously identified as being associated with iron metabolism or homeostasis, including those identified in

Tables 2 and 4. In particular embodiments, the polynucleotide or polypeptide is a TSC22D1 polynucleotide or polypeptide.

B. Kits and Arrays for the Detection of Iron-Related Disorders

The present invention also encompasses kits for detecting the presence of an iron-related disorder in a patient. For example, the kit can comprise one or more compounds or agents capable of detecting an iron-related polypeptide or mRNA in a biological sample, such as oligonucleotides and antibodies. In certain embodiments, the antibody is labeled. The kit may optionally include one or more control samples. The compounds or agents can be packaged in a suitable container. The kit can further comprise instructions for using the kit. In particular embodiments, the kit or array comprises an oligonucleotide or antibody (or fragment thereof) that specifically bind to a Tsc22d1 polynucleotide or polypeptide.

Another embodiment of the present invention relates to a plurality of polynucleotides for detecting the expression of one or more iron-related genes described herein. The plurality of polynucleotides consists of, or consists essentially of, at least two polynucleotide probes that are complementary to RNA transcripts, or nucleotides derived therefrom, of at least one gene that has been identified herein as being selectively regulated in patients having an iron-related disorder, and is therefore distinguished from previously known nucleic acid arrays and primer sets. The plurality of polynucleotides within the above-limitation includes at least two or more polynucleotide probes (*e.g.*, at least 2, 3, 4, 5, 6, and so on, in whole integer increments, up to all of the possible probes) that are complementary to RNA transcripts, or nucleotides derived therefrom, of at least one gene, and preferably, at least two or more genes identified by the present inventors. Such genes are selected from any of the genes listed in the tables provided herein and can include any number of genes, in whole integers (*e.g.*, 1, 2, 3, 4, . . .). Multiple probes can also be used to detect the same gene or to detect different splice variants of the same gene. In one aspect, each of the polynucleotides in the plurality is at least five nucleotides in length. In one aspect, the plurality of polynucleotides consists of at least two polynucleotides, wherein each polynucleotide is at least five nucleotides in length, and wherein each polynucleotide is complementary to an RNA transcript, or nucleotide derived therefrom, of a gene comprising, or expressing a transcript comprising, a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-48 and 98-178. In another aspect, the plurality of polynucleotides comprises polynucleotides that are complementary to an RNA transcript, or a nucleotide derived therefrom, of

at least two genes comprising, or expressing a transcript comprising, a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-48 and 98-178. In another aspect, the plurality of polynucleotides comprises polynucleotides that are complementary to an RNA transcript, or a nucleotide derived therefrom, of at least five genes, at least 10 genes, or up to all of the genes, comprising, or expressing a transcript comprising, a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-48 and 98-178. In particular embodiments, the plurality of polynucleotides comprises a Tsc22d1 polynucleotide.

Another embodiment of the present invention relates to a plurality of antibodies, or antigen binding fragments thereof, for the detection of the expression of iron-related polypeptides. The plurality of antibodies, or antigen binding fragments thereof, consists of antibodies, or antigen binding fragments thereof, that selectively bind to polypeptide encoded by genes found to have altered expression in iron-related disorders. In addition, the plurality of antibodies, or antigen binding fragments thereof, comprises antibodies, or antigen binding fragments thereof, that selectively or specifically bind to proteins or portions thereof (peptides) encoded by any of the genes from the tables provided herein. In one aspect, the plurality of antibodies, antigen binding fragments thereof, or antigen binding peptides consists of at least two antibodies, antigen binding fragments thereof, or antigen binding peptides, each of which selectively binds to a protein encoded by a gene comprising, or expressing a transcript comprising, a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-48 and 98-178. In another embodiment, a plurality of antibodies, antigen binding fragments thereof, or antigen binding peptides consist of at least two antibodies, antigen binding fragments thereof, or antigen binding peptides, each of which selectively binds to a secreted protein, including any combination of the proteins provided in Table 4. In one embodiment, the plurality of antibodies comprises an antibody, or fragment thereof, that specifically binds to a TSC22D1 polypeptide.

According to the present invention, a plurality of antibodies, or antigen binding fragments thereof, refers to at least 2, and more preferably at least 3, and more preferably at least 4, and more preferably at least 5, and more preferably at least 6, and more preferably at least 7, and more preferably at least 8, and more preferably at least 9, and more preferably at least 10, and so on, in increments of one, up to any suitable number of antibodies, or antigen binding fragments thereof, including antibodies representing all of the genes described herein, or antigen binding fragments thereof.

The present invention further comprises arrays comprising a plurality of oligonucletoides or antibodies that bind to iron-related polynucleotides or polypeptides described herein. In particular embodiments, the oligonucleotides or antibodies are labeled. In certain embodiments, the arrays further comprise one or more control oligonucleotides or antibodies.

C. Methods of Treating Iron-Related Disorders

The genes described herein are predicted to have direct relevance as clinical modifiers of iron balance or homeostasis, based upon the discovery of the relationship between their expression levels and iron overload. In addition, given their apparent role in regulating iron homeostasis, these genes present novel targets for the treatment of iron related disorders. Accordingly, the present invention includes methods of modulating the expression or activity of one or more of the iron-related genes and proteins described in Tables 2-4, which methods may be used to regulate iron homeostasis and/or treat or prevent an iron-related disorder described herein. In certain embodiments, these methods involve restoring the expression or activity of one or more genes or polypeptides identified as having altered expression in an iron-related disease to a level closer to normal. Accordingly, this may involve increasing the level of expression or activity of a gene or polypeptide having a reduced level in an iron-related disease or disorder, or it may involve decreasing the level of expression or activity of a gene or polypeptide having an increased level in an iron-related disease or disorder.

As noted above, in various embodiments, methods of modulating the biological activity or expression of an iron-related gene or polypeptide include either increasing the activity or expression of an iron-related gene or polypeptide or decreasing the activity or expression of an iron-related gene or polypeptide. This may be accomplished genetically, *e.g.*, by introducing a transgene encoding an iron-related polypeptide into a cell or animal or by mutating or disrupting an iron-related gene in a cell or animal. This may also be accomplished using compounds that bind to iron-related polypeptides or polynucleotides, or bind to another molecule that regulates the expression or activity of an iron-related polynucleotide or polypeptide, *e.g.*, compounds that bind to a ligand of an iron-related polypeptide or a transcriptional repressor that binds the promoter of an iron-related gene. Accordingly, the methods of the present invention may directly affect iron-related polynucleotide or polypeptide activity or expression, or they may indirectly affect iron-related polynucleotide or polypeptide activity or expression.

Methods that involve increasing activity or expression of an iron-related polynucleotide or polypeptide are particularly useful in treating or preventing disease states or conditions characterized by insufficient levels of an iron-related polynucleotide or polypeptide. Methods that involve decreasing activity or expression are particularly useful in treating or preventing disease states or conditions characterized by excessive levels of an iron-related polynucleotide or polypeptide. These methods may involve administering a compound that modulates an iron-related polynucleotide or polypeptide, wherein the compound is either an agonist or antagonist of an iron-related polynucleotide or polypeptide, to a cell or patient.

In one embodiment, the present invention includes a method of treating or preventing an iron-related disorder in a mammal, comprising administering a polynucleotide that encodes an iron-related polypeptide, or a functional variant or fragment thereof, to said mammal, wherein said polynucleotide is expressed in said mammal. In certain embodiments, the polynucleotide is an expression vector, and in other embodiments, the polynucleotide is a transgene. In one embodiment, the polynucleotide is a Tsc22d1 polynucleotide or encodes a TSC22D1 polypeptide.

In another embodiment, the present invention includes a method of treating or preventing an iron-related disorder in a mammal, comprising administering an iron-related polypeptide, or a functional variant or fragment thereof, to said mammal. In one embodiment, the polypeptide is a TSC22D1 polypeptide or functional variant or fragment thereof.

In one embodiment, the present invention includes a method of treating or preventing an iron-related disorder in a mammal, comprising administering a polynucleotide comprising a sequence identical to a region of polynucleotide sequence set forth in SEQ ID NOs1-48 and 98-178, or a complement thereof, to said mammal, wherein said polynucleotide inhibits expression of an iron-related polynucleotide or polypeptide. In certain embodiments, the polynucleotide is an expression vector, and in particular embodiments, the polynucleotide is an antisense RNA, an RNAi molecule, or a ribozyme. In one embodiment, the polynucleotide comprises a Tsc22d1 polynucleotide or fragment thereof, or complement of either.

In another embodiment, the present invention includes a method of treating or preventing an iron-related disorder in a mammal, comprising administering an antibody that specifically binds to an iron-related polypeptide having an sequence set forth in SEQ ID NOs:49-97 and 179-253 to said mammal.

In one embodiment, the antibody inhibits a biological activity of an iron-related polypeptide, while in another embodiment, the antibody increases a biological activity of an iron-related polypeptide. In one embodiment, the antibody specifically bind to a TSC22D1 polypeptide.

The present invention further includes a method of treating or preventing an iron-related disorder in a mammal, comprising administering a small molecule that specifically binds to an iron-related polypeptide having the sequence set forth in SEQ ID NOs:49-97 and 179-253 to said mammal. In one embodiment, the small molecule inhibits a biological activity of an iron-related polypeptide, while in another embodiment, the small molecule increases a biological activity of an iron-related polypeptide. In one embodiment, the small molecule binds to a TSC22D1 polypeptide.

In particular embodiments of the methods of treating or preventing an iron-related disease or disorder associated with increased iron levels or iron overload, a patient is administered an agent that increased the amount or activity of a TSC22D1 polypeptide. In various embodiments, this may be, *e.g.*, a polynucleotide that encodes a TSC22D1 polypeptide or functional variant or fragment thereof, a TSC22D1 polypeptide or functional variant or fragment thereof, an antibody that agonizes TSC22D1 activity, or a compound that agonizes TSC22D1 activity.

In particular embodiments of the methods of treating or preventing an iron-related disease or disorder associated with decreased iron levels, such as AI, a patient is administered an agent that decreased the amount or activity of a TSC22D1 polypeptide. In various embodiments, this may be, e.g., a dominant-negative TSC22D1 polypeptide, an inhibitory polynucleotide that specifically bind to a Tsc22d1 polynucleotide, comprising a portion of a Tsc22d1 polynucleotide, such as an siRNA, antisense RNA, ribozyme, or microRNA.

The methods of the present invention may be practiced using a variety of different compounds that modulate an activity or expression of an iron-related polynucleotide or polypeptide ("modulators"). In particular embodiments, modulators are polynucleotides, polypeptides, peptides, peptide nucleic acids, antibodies and fragments thereof, viruses, small molecules, inorganic compounds and organic compounds. Examples of modulators that increase expression include iron-related polypeptides and polynucleotides, such as transgenes and expression vectors. Examples of modulators that decrease expression include, *e.g.*, knockout constructs, antisense RNA, and RNAi molecules. Examples of modulators that decrease activity include compounds that interfere with binding to

a ligand or inhibit downstream signaling events, such as antagonist antibodies to iron-related polypeptides. Examples of modulators that increase activity include compounds that enhance binding to a ligand or promote downstream signaling events, such as agonist antibodies to iron-related polypeptides.

1. Polypeptides and Polynucleotides

In certain embodiments, methods of the invention are practiced using peptide or polypeptide modulators of iron-related polynucleotides or polypeptides. In certain embodiments, the modulator is a peptide or polypeptide comprising amino acid sequence identical to or substantially identical to a portion of an iron-related polypeptide. The amino acid sequences of iron-related polypeptides of the present invention are provided in SEQ ID NOs:49-97 and 179-253. Thus, in certain embodiments, the polypeptide modulator may be a fragment of an iron-related polypeptide, and preferably a functional fragment, which has one or more biological activities in common with a full length iron-related polypeptide, *e.g.*, binding to a ligand or stimulation of a downstream signaling pathway.

Peptides and polypeptides may be readily synthesized or produced recombinantly using routine methods known and available in the art. For example, iron-related polynucleotides can be used as a tool to express an iron-related polypeptide in an appropriate cell *in vitro* or *in vivo* (gene therapy). Methods well known to those skilled in the art may be used to construct expression vectors containing sequences encoding a polynucleotide or polypeptide of interest and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook, J. *et al.* (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., and Ausubel, F. M. *et al.* (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York. N.Y.

In one embodiment, expression of an iron-related polypeptide is increased using an expression construct that expresses a full length iron-related polypeptide, or a functional variant or fragment thereof. In various embodiments, the expression construct is adapted for transient expression in a cell, while in other embodiments, the expression construct is adapted for stable expression in a cell. Accordingly, in certain embodiments, the expression construct may be a plasmid or virus.

In another embodiments, expression of an iron-related polypeptide is increased by inserting a transgene encoding an iron-related polypeptide into an

animal. A variety of vectors and constructs suitable for introducing a transgene into an animal's genome have been described and demonstrated to successfully deliver therapeutic levels of a polypeptide to a cell.

In one embodiment, expression constructs of the invention comprise polynucleotide sequences encoding all or a region of a modulator, in addition to regulatory sequences that govern expression of coding sequences. Regulatory sequences present in an expression vector include those non-translated regions of the vector, *e.g.,* enhancers, promoters, 5' and 3' untranslated regions, which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and cell utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. A variety of expression systems may be employed for the recombinant production of polypeptides, including, *e.g.*, baculovirus, herpes virus, adenovirus, adeno-associated virus, bacterial systems, and eukaryotic systems such as CHO cells. Naked DNA and DNA-liposome complexes can also be used. In particular embodiments, polynucleotides and polypeptides may be introduced into cells in plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

In another embodiment, the activity of an iron-related polypeptide is altered by over expression of a dominant negative inhibitor of an iron-related polypeptide. Dominant negative inhibitors of iron-related polypeptides are typically mutant forms of iron-related polypeptides, which reduce or block the activity of a wild type iron-related polypeptide, *e.g.*, by competing for binding to a ligand but failing to fully activate the iron-related polypeptide signaling pathway.

Polypeptide inhibitors also include other variants and fragments of iron-related polypeptides having reduced biological activity as compared to wild type iron-related polypeptides. In certain embodiments, polypeptide inhibitors are soluble variants or fragments of an iron-related polypeptide.

Various polynucleotides are contemplated for use as modulators of iron-related polynucleotide or polypeptide expression and/or activity. In one embodiment, a polynucleotide encoding an iron-related polypeptide, or a functional variant or fragment thereof, is used to increase expression of an iron-related polynucleotide or polypeptide, essentially as described above. The polynucleotide sequence of iron-related polynucleotides of the present invention are provided in SEQ ID NOs:1-48 and 98-178. In certain embodiments, polynucleotides include

expression vectors and replacement or insertion vectors designed for integration into the genome of a cell, and suitable for gene therapy, *e.g.*, transgenes, or disruption of a iron-related gene allele, *e.g.*, knockout constructs.

In certain embodiments, polynucleotide inhibitors of expression of an iron-related polynucleotide or polypeptide are double-stranded or single-stranded DNA or RNA, including, *e.g.*, antisense RNA, ribozymes, or RNA interference reagents designed to specifically target iron-related polynucleotides, according to methods known and available in the art. Polynucleotide inhibitors may also be DNA-RNA hybrids. Other polynucleotide inhibitors include, *e.g.,* targeting vectors designed for integration into the genome and suitable for deleting all or a portion of an iron-related gene allele or an iron-related gene allele, *e.g.,* through insertional mutagenesis.

In one embodiment, an inhibitor of expression of an iron-related polynucleotide is an antisense RNA directed to an iron-related polynucleotide, or another components of its signaling cascade or biological pathway. Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal $GABA_A$ receptor and human EGF (Jaskulski *et al.*, Science. 1988 Jun 10;240(4858):1544-6; Vasanthakumar and Ahmed, Cancer Commun. 1989;1(4):225-32; Peris *et al.*, Brain Res Mol Brain Res. 1998 Jun 15;57(2):310-20; U. S. Patent 5,801,154; U.S. Patent 5,789,573; U. S. Patent 5,718,709 and U.S. Patent 5,610,288). Furthermore, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.* cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683).

Therefore, in certain embodiments, the present invention relates to methods of modulating the expression of iron-related polynucleotides or polypeptides comprising providing oligonucleotide sequences that comprise all, or a portion of, any sequence that is capable of specifically binding to a target iron-related polynucleotide sequence, or a complement thereof. In another embodiment, the oligonucleotide sequence comprises all, or a portion of an iron-

related polynucleotide sequence set forth in SEQ ID NOs:1-48 and 98-178, or a complement thereof. In one embodiment, the antisense oligonucleotide comprises DNA or derivatives thereof. In another embodiment, the oligonucleotide comprises RNA or derivatives thereof. The antisense oligonucleotides may be modified DNAs comprising a phosphorothioated modified backbone. Also, the oligonucleotide sequences may comprise peptide nucleic acids or derivatives thereof. In each case, preferred compositions comprise a sequence region that is complementary, and more preferably, completely complementary to one or more portions of a target iron-related gene or polynucleotide sequence.

Methods of producing antisense molecules are known in the art and can be readily adapted to produce an antisense molecule that targets an iron-related polynucleotide. Selection of antisense compositions specific for a given sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense compositions may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.,* Nucleic Acids Res. 1997, 25(17):3389-402).

According to another embodiment of methods of the invention, ribozyme molecules are used to inhibit expression of a target iron-related gene or polynucleotide sequence. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (Kim and Cech, Proc. Natl. Acad. Sci. U S A. 1987 Dec;84(24):8788-92; Forster and Symons, Cell. 1987 Apr 24;49(2):211-20). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, Cell. 1981 Dec;27(3 Pt 2):487-96; Michel and Westhof, J. Mol. Biol. 1990 Dec 5;216(3):585-610; Reinhold-Hurek and Shub, Nature. 1992 May 14;357(6374):173-6). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

At least six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described by Rossi *et al.* Nucleic Acids Res. 1992 Sep 11;20(17):4559-65. Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz, Biochemistry 1989 Jun 13;28(12):4929-33; Hampel *et al.,* Nucleic Acids Res. 1990 Jan 25;18(2):299-304 and U. S. Patent 5,631,359. An example of the hepatitis δ virus motif is described by Perrotta and Been, Biochemistry. 1992 Dec 1;31(47):11843-52; an example of the RNaseP motif is described by Guerrier-Takada *et al.*, Cell. 1983 Dec;35(3 Pt 2):849-57; Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, Cell. 1990 May 18;61(4):685-96; Saville and Collins, Proc Natl Acad Sci U S A. 1991 Oct 1;88(19):8826-30; Collins and Olive, Biochemistry. 1993 Mar 23;32(11):2795-9); and an example of the Group I intron is described in U. S. Patent 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus, the ribozyme constructs need not be limited to specific motifs mentioned herein.

Methods of producing a ribozyme targeted to an iron-related polynucleotide are known in the art. Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595,

each specifically incorporated herein by reference, and synthesized to be tested *in vitro* and *in vivo*, as described therein.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U. S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

RNA interference methods using RNAi molecules also may be used to disrupt the expression of a gene or polynucleotide of interest, including an iron-related gene or another gene associated with its signaling cascade or biological pathway. While the first described RNAi molecules were RNA:RNA hybrids comprising both an RNA sense and an RNA antisense strand, it has now been demonstrated that DNA sense:RNA antisense hybrids, RNA sense:DNA antisense hybrids, and DNA:DNA hybrids are capable of mediating RNAi (Lamberton, J.S. and Christian, A.T., (2003) Molecular Biotechnology 24:111-119). Accordingly, the invention includes the use of RNAi reagents comprising any of these different types of double-stranded molecules. In addition, it is understood that RNAi reagents may be used and introduced to cells in a variety of forms. Accordingly, as used herein, RNAi reagents encompasses any and all reagents capable of inducing an RNAi response in cells, including, but not limited to, double-stranded polynucleotides comprising two separate strands, *i.e.* a sense strand and an antisense strand, polynucleotides comprising a hairpin loop of complementary sequences, which forms a double-stranded region, *e.g.*, shRNAi molecules, and expression vectors that express one or more polynucleotides capable of forming a double-stranded polynucleotide alone or in combination with another polynucleotide.

In one particular embodiment, a dsRNA molecule that targets and induces degradation of an iron-related polynucleotide is introduced to a cell. While the exact mechanism is not essential to the invention, it is believed the association of the dsRNA to the target gene is defined by the homology between the dsRNA and the actual and/or predicted mRNA transcript. It is believed that this association will affect the ability of the dsRNA to disrupt the target gene. DsRNA methods and reagents are described in PCT applications WO 99/32619, WO

01/68836, WO 01/29058, WO 02/44321, WO 01/92513, WO 01/96584, and WO 01/75164, which are hereby incorporated by reference in their entirety.

In one embodiment of the invention, RNA interference (RNAi) may be used to specifically inhibit expression of an iron-related polynucleotide. Double-stranded RNA-mediated suppression of gene and nucleic acid expression may be accomplished according to the invention by introducing dsRNA, siRNA or shRNA into cells or organisms. SiRNA may be double-stranded RNA, or a hybrid molecule comprising both RNA and DNA, *e.g.*, one RNA strand and one DNA strand. It has been demonstrated that the direct introduction of siRNAs to a cell can trigger RNAi in mammalian cells (Elshabir, S.M., *et al.* Nature 411:494-498 (2001)). Furthermore, suppression in mammalian cells occurred at the RNA level and was specific for the targeted genes, with a strong correlation between RNA and protein suppression (Caplen, N. *et al.*, Proc. Natl. Acad. Sci. USA 98:9746-9747 (2001)). In addition, it was shown that a wide variety of cell lines, including HeLa S3, COS7, 293, NIH/3T3, A549, HT-29, CHO-KI and MCF-7 cells, are susceptible to some level of siRNA silencing (Brown, D. *et al.* TechNotes 9(1):1-7, available at http://www.dot.ambion.dot.com/techlib/tn/91/912.html (9/1/02)).

RNAi reagents targeting iron-related polynucleotides can be readily prepared according to procedures known in the art. Structural characteristics of effective siRNA molecules have been identified. Elshabir, S.M. *et al.* (2001) Nature 411:494-498 and Elshabir, S.M. *et al.* (2001), EMBO 20:6877-6888. Accordingly, one of skill in the art would understand that a wide variety of different siRNA molecules may be used to target a specific gene or transcript. In certain embodiments, siRNA molecules according to the invention are 16 - 30 or 18 - 25 nucleotides in length, including each integer in between. In one embodiment, an siRNA is 21 nucleotides in length. In certain embodiments, siRNAs have 0-7 nucleotide 3' overhangs or 0-4 nucleotide 5' overhangs. In one embodiment, an siRNA molecule has a two nucleotide 3' overhang. In one embodiment, an siRNA is 21 nucleotides in length with two nucleotide 3' overhangs (*i.e.* they contain a 19 nucleotide complementary region between the sense and antisense strands). In certain embodiments, the overhangs are UU or dTdT 3' overhangs. Generally, siRNA molecules are completely complementary to one strand of a target DNA molecule, since even single base pair mismatches have been shown to reduce silencing. In other embodiments, siRNAs may have a modified backbone composition, such as, for example, 2'-deoxy- or 2'-O-methyl modifications. However, in preferred embodiments, the entire strand of the siRNA is not made with either 2' deoxy or 2'-O-modified bases.

In one embodiment, siRNA target sites are selected by scanning the target mRNA transcript sequence for the occurrence of AA dinucleotide sequences. Each AA dinucleotide sequence in combination with the 3' adjacent approximately 19 nucleotides are potential siRNA target sites. In one embodiment, siRNA target sites are preferentially not located within the 5' and 3' untranslated regions (UTRs) or regions near the start codon (within approximately 75 bases), since proteins that bind regulatory regions may interfere with the binding of the siRNP endonuclease complex (Elshabir, S. *et al.* Nature 411:494-498 (2001); Elshabir, S. *et al.* EMBO J. 20:6877-6888 (2001)). In addition, potential target sites may be compared to an appropriate genome database, such as BLASTN 2.0.5, available on the NCBI server at www.ncbi.nlm, and potential target sequences with significant homology to other coding sequences eliminated.

Short hairpin RNAs may also be used to inhibit or knockdown gene or nucleic acid expression according to the invention. Short Hairpin RNA (shRNA) is a form of hairpin RNA capable of sequence-specifically reducing expression of a target gene. Short hairpin RNAs may offer an advantage over siRNAs in suppressing gene expression, as they are generally more stable and less susceptible to degradation in the cellular environment. It has been established that such short hairpin RNA-mediated gene silencing (also termed SHAGging) works in a variety of normal and cancer cell lines, and in mammalian cells, including mouse and human cells. Paddison, P. *et al.*, Genes Dev. 16(8):948-58 (2002). Furthermore, transgenic cell lines bearing chromosomal genes that code for engineered shRNAs have been generated. These cells are able to constitutively synthesize shRNAs, thereby facilitating long-lasting or constitutive gene silencing that may be passed on to progeny cells. Paddison, P. *et al.*, Proc. Natl. Acad. Sci. USA 99(3):1443-1448 (2002).

ShRNAs contain a stem loop structure. In certain embodiments, they may contain variable stem lengths, typically from 19 to 29 nucleotides in length, or any number in between. In certain embodiments, hairpins contain 19 to 21 nucleotide stems, while in other embodiments, hairpins contain 27 to 29 nucleotide stems. In certain embodiments, loop size is between 4 to 23 nucleotides in length, although the loop size may be larger than 23 nucleotides without significantly affecting silencing activity. ShRNA molecules may contain mismatches, for example G-U mismatches between the two strands of the shRNA stem without decreasing potency. In fact, in certain embodiments, shRNAs are designed to include one or several G-U pairings in the hairpin stem to stabilize hairpins during propagation in bacteria, for example. However, complementarity between the

portion of the stem that binds to the target mRNA (antisense strand) and the mRNA is typically required, and even a single base pair mismatch is this region may abolish silencing. 5' and 3' overhangs are not required, since they do not appear to be critical for shRNA function, although they may be present (Paddison *et al.* (2002) Genes & Dev. 16(8):948-58).

In certain embodiments, the activity of an iron-related polypeptide is altered by mutating a gene encoding the iron-related polypeptide or a gene encoding another component of its biological pathway. A variety of methods of mutating an endogenous gene are known and available in the art, including, *e.g.*, insertional mutagenesis and knockout methods, such as those described herein. Accordingly, the invention includes methods of knocking out one or more alleles of an iron-related gene. It is understood that knockout vectors according to the invention include any vector capable of disrupting expression or activity of an iron-related gene, including, in certain embodiments, both gene trap and targeting vectors.

In preferred methods, targeting vectors are used to selectively disrupt an iron-related gene. Knockout vectors of the invention include those that alter gene expression, for example, by disrupting a regulatory element of the gene, including, *e.g.,* inserting a regulatory element that reduces gene expression or deleting or otherwise reducing the activity of an endogenous element that positively affects transcription of the target gene. In other embodiments, knockout vectors of the invention disrupt, *e.g.*, delete or mutate, the 5' region, 3' region or coding region of the gene. In some embodiments, knockout vectors delete a region or the entirety of the coding region of the gene. In certain embodiments, knockout vectors delete a region of the gene, while in other embodiments, they insert exogenous sequences into the gene. In addition, in certain embodiments, including those using replacement vectors, knockout vectors both remove a region of a gene and introduce an exogenous sequence.

Targeting vectors of the invention include all vectors capable of undergoing homologous recombination with an endogenous iron-related gene, including replacement vectors. Targeting vectors include all those used in methods of positive selection, negative selection, positive-negative selection, and positive switch selection. Targeting vectors employing positive, negative, and positive-negative selection are well known in the art and representative examples are described in Joyner, A.L., Gene Targeting: A practical Approach, 2nd ed. (2000) and references cited therein.

2. Antibodies

Antibodies, or antigen-binding fragments thereof, that specifically bind iron-related polypeptides are also modulators, *i.e.*, activators or inhibitors, of iron-related polypeptide according to the methods described herein. An antibody, or antigen-binding fragment thereof, is said to "specifically bind," "immunologically bind," and/or is "immunologically reactive" to a polypeptide of the invention if it reacts at a detectable level (within, for example, an ELISA assay) with the polypeptide, and does not react detectably with unrelated polypeptides under similar conditions. Antibodies are considered to specifically bind to a target polypeptide when the binding affinity is at least $1x10^{-7}$ M or, preferably, at least $1x10^{-8}$ M. In certain embodiments, an antibody specifically binds a polypeptide having a sequence set forth in any one of SEQ ID NOs:49-97 and 179-253.

Antibodies used in the methods of the invention include, but are not limited to, polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, fully human antibodies, Primatized® antibodies, single chains, Fab fragments and scFv fragments.

Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.,* Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies via conventional techniques known in the art, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of recombinant antibodies. Monoclonal antibodies specific for an antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, Eur. J. Immunol. *6*:511-519, 1976, and improvements thereto. Methods of making chimeric and humanized antibodies are well known in the art, (See, *e.g.*, U.S. Pat. No. 4,816,567, International Application No. WO84/03712, respectively).

In certain embodiment, methods of preparing monoclonal antibodies involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a

selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

A number of therapeutically useful molecules are known in the art, which comprise antigen-binding sites that are capable of exhibiting immunological binding properties of an antibody molecule. The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the "F(ab)" fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the "$F(ab')_2$ " fragment which comprises both antigen-binding sites. An "Fv" fragment can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes a non-covalent $V_H::V_L$ heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. Inbar et al. (1972) Proc. Nat. Acad. Sci. USA 69:2659-2662; Hochman et al. (1976) Biochem. 15:2706-2710; and Ehrlich et al. (1980) Biochem. 19:4091-4096.

A single chain Fv ("sFv") polypeptide is a covalently linked $V_H::V_L$ heterodimer which is expressed from a gene fusion including V_H- and V_L-encoding genes linked by a peptide-encoding linker. Huston et al. (1988) Proc. Nat. Acad. Sci. USA 85(16):5879-5883. A number of methods have been described to discern chemical structures for converting the naturally aggregated--but chemically separated--light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar

to the structure of an antigen-binding site. See, *e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston et al.; and U.S. Pat. No. 4,946,778, to Ladner et al.

In certain embodiments, each of the above-described molecules includes a heavy chain and a light chain CDR set, respectively interposed between a heavy chain and a light chain FR set which provide support to the CDRS and define the spatial relationship of the CDRs relative to each other. As used herein, the term "CDR set" refers to the three hypervariable regions of a heavy or light chain V region. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (*e.g.*, a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site.

As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRS. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures--regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

The invention further includes veneered framework (FR) antibodies. As used herein, the terms "veneered FRs" and "recombinantly veneered FRs" refer to the selective replacement of FR residues from, *e.g.*, a rodent heavy or light chain V region, with human FR residues in order to provide a xenogeneic molecule comprising an antigen-binding site which retains substantially all of the native FR polypeptide folding structure. Veneering techniques are based on the

understanding that the ligand binding characteristics of an antigen-binding site are determined primarily by the structure and relative disposition of the heavy and light chain CDR sets within the antigen-binding surface. Davies et al. (1990) Ann. Rev. Biochem. 59:439-473. Thus, antigen binding specificity can be preserved in a humanized antibody only wherein the CDR structures, their interaction with each other, and their interaction with the rest of the V region domains are carefully maintained. By using veneering techniques, exterior (*e.g.*, solvent-accessible) FR residues which are readily encountered by the immune system are selectively replaced with human residues to provide a hybrid molecule that comprises either a weakly immunogenic, or substantially non-immunogenic veneered surface.

Fab or $F(ab')_2$ fragments may be wholly animal or human derived, or they may be in chimeric form, such that the constant domains are derived from the constant regions of human immunoglobulins and the variable regions are derived from the parent murine MAb. Alternatively, the Fv, Fab, or $F(ab')_2$ may be humanized, so that only the complementarity determining regions (CDR) are derived from an animal MAb, and the constant domains and the framework regions of the variable regions are of human origin. These chimeric and humanized fragments are less immunogenic than their wholly animal counterparts, and thus more suitable for in vivo use, especially over prolonged periods.

A number of "humanized" antibody molecules comprising an antigen-binding site derived from a non-human immunoglobulin have been described, including chimeric antibodies having rodent V regions and their associated CDRs fused to human constant domains (Winter et al. (1991) Nature 349:293-299; Lobuglio et al. (1989) Proc. Nat. Acad. Sci. USA 86:4220-4224; Shaw et al. (1987) J. Immunol. 138:4534-4538; and Brown et al. (1987) Cancer Res. 47:3577-3583), rodent CDRs grafted into a human supporting FR prior to fusion with an appropriate human antibody constant domain (Riechmann et al. (1988) Nature 332:323-327; Verhoeyen et al. (1988) Science 239:1534-1536; and Jones et al. (1986) Nature 321:522-525), and rodent CDRs supported by recombinantly veneered rodent FRs (European Patent Publication No. 519,596, published Dec. 23, 1992). These "humanized" molecules are designed to minimize unwanted immunological response toward rodent antihuman antibody molecules which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients.

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid

residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims *et al.*, J. Immunol., 151:2296 (1993); Chothia *et al.*, J. Mol. Biol., 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta *et al.*, J. Immunol., 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences

so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al.*, Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits *et al.*, Nature, 362:255-258 (1993); Bruggermann *et al.*, Year in Immuno., 7:33 (1993); and U.S. Pat. Nos. 5,591,669, 5,589,369 and 5,545,807.

In one embodiment, humanized or fully human antibodies of the present invention are prepared according to the methods described in U.S. Patent Nos. 5,770,429, 5,833,985, 5,837,243, 5,922,845, 6,071,517, 6,096,311, 6,111,166, 6,270,765, 6,303,755, 6,365,116, 6,410,690, 6,682,928, and 6,984,720, all assigned to Medarex, Inc.

Alternatively, phage display technology (McCafferty *et al.*, Nature 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review see, *e.g.*, Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a

diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, J. Mol. Biol. 222:581-597 (1991), or Griffith *et al.*, EMBO J. 12:725-734 (1993). See, also, U.S. Pat. Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by *in vitro* activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

Antibodies to iron-related polypeptides may be agonists that increase an activity of an iron-related polypeptide, or they may be antagonists that decrease an activity of an iron-related polypeptide. In one embodiment, an antibody serves as an inhibitor of iron-related polypeptide signaling by binding to an iron-related polypeptide, *e.g.,* the extracellular domain, and thereby inhibiting binding of a ligand to the iron-related polypeptide.

3. Small Molecules

Modulators (inhibitors or activators) of the present invention further include large or small inorganic or organic molecules. In certain embodiments, modulators are small organic molecules, or derivatives or analogs thereof. Such small molecules preferably have a molecular weight below 2,000 daltons, more preferably between 300 and 1,000 daltons, and most preferably between 400 and 700 daltons. It is preferred that these small molecules be organic molecules.

In certain embodiments, a modulator includes a protecting group. The term "protecting group" refers to chemical moieties that block at least some reactive moieties and prevent such groups from participating in chemical reactions until the protective group is removed (or "cleaved"). Examples of blocking/protecting groups are described, *e.g.,* in Greene and Wuts, Protective Groups in Organic Synthesis, 3rd Ed., John Wiley & Sons, New York, NY, 1999.

Any of the modulators may possess one or more chiral centers and each center may exist in the R or S configuration. Modulators of the present invention include all diastereomeric, enantiomeric, and epimeric forms as well as mixtures thereof. Stereoisomers may be obtained, if desired, by methods known in the art as, for example, the separation of stereoisomers by

chiral chromatographic columns. Modulators further include of *N*-oxides, crystalline forms (also known as polymorphs), and pharmaceutically acceptable salts, as well as active metabolites of any inhibitor. All tautomers are included within the scope of the modulators presented herein. In addition, the modulators described herein can exist in unsolvated as well as solvated forms with pharmaceutically acceptable solvents such as water, ethanol, and the like. The solvated forms of the modulators presented herein are also included within the present invention.

In a particular embodiment, a small molecule inhibitor binds to an iron-related polypeptide. In one embodiment, a small molecule binds to the extracellular region of an iron-related polypeptide and interferes or reduces ligand binding to the iron-related polypeptide.

Modulators of iron-related polypeptides and polynucleotides, including small organic compounds, may be identified according to routine screening procedures available in the art, *e.g.*, using commercially available libraries of such compounds.

4. Pharmaceutical Compositions and Administration

Compounds of the invention may be administered in a pharmaceutical composition. Pharmaceutical compositions of the present invention comprise a compound that modulates the activity or expression of an iron-related polypeptide and a pharmaceutically-acceptable diluent, carrier, or excipient. In one embodiment, such compounds may comprise a polynucleotide or polypeptide sequence set forth in any one of SEQ ID NOs:1-232, or a complement or fragment thereof. Pharmaceutical compositions may be administered in unit dosage form.

Any appropriate route of administration may be employed, for example, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraarticular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Examples of specific routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration.

Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients.

Methods well known in the art for making pharmaceutical compositions and formulations are found in, for example, Remington: The Science and Practice of Pharmacy, (20th ed.) ed. A. R. Gennaro A R., 2000, Lippincott: Philadelphia. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for copounds of the invention include ethylenevinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, or example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

Therapeutic formulations may be in the form of liquid solutions or suspension; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS).

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be

incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

Liposomal suspensions (including liposomes targeted to specific cell types) can also be used as pharmaceutically acceptable carriers. A variety of liposomal formulations suitable for delivering a compound to an animal have been described and demonstrated to be effective in delivering a variety of compound, including, *e.g.,* small molecules, nucleic acids, and polypeptides.

It may be advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Depending on the type and severity of the disease, about 1 ug/Kg to 15 mg/Kg (*e.g.*, 0.1 to 20 mg/Kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 ug/Kg to 100 mg/Kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until

a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy can be monitored by standard techniques and assays. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

Data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

The present invention encompasses compounds that modulate expression or activity. A compound may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e. including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention.

Appropriate doses of a small molecule also depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

D. Methods of Identifying Modulators of Iron Homeostasis Useful in the Treatment of Iron Disorders

The invention further provides methods of identifying and producing compounds useful in the treatment or prevention of iron-related disorders. These

compounds include modulators (*i.e.*, inhibitors and inducers, including antagonists and agonists) of the expression and/or activity of an iron-related polynucleotide or polypeptide. In general, such modulators are identified by screening candidate molecules, including, *e.g.*, all of the different types of molecules described above. Any assay suitable for determining expression or activity of an iron-related polynucleotide or polypeptide may be utilized, including, but not limited to, binding assays and biological functional assays described herein. The invention contemplates at least two different types of inhibitors, including (1) compounds that decrease a functional activity of an iron-related polypeptide; and (2) compounds that decrease expression levels of an iron-related polynucleoide or polypeptide. An inhibitor is identified as a compound that reduces one or more activities or expression of an iron-related polynucleotide or polypeptide by at least 10%, at least 25%, at least 50%, at least 75%, or 100%.

In general, the invention contemplates two different types of inducers, including (1) compounds that increase the functional activity of an iron-related polypeptide; and (2) compounds that increase expression levels of an iron-related polynucleotide or polypetide, including, *e.g.*, an expression construct. In one embodiment, an inducer is identified as a molecule or compound that increases one or more activities by at least two-fold, at least five-fold, at least ten-fold or more. In the context of overexpression, an inducer is a molecule or compound that increases expression at least two-fold, at least five-fold, at least ten-fold or more.

In particular embodiments, the methods of the present invention are practiced to identify a modulator of a TSC22D1 polypeptide.

Modulators may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, collections of chemical compounds, and natural product mixtures. They may be identified using any of the numerous approaches in combinatorial library methods known in the art, including, *e.g.*, screening of biological libraries, screening of spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the `one-bead one-compound` library method, and synthetic library methods using affinity chromatography selection.

Candidate modulators may be screened individually, *e.g.*, when a specific molecule is predicted to function as an inhibitor or inducer/activator. Candidate modulators may be purified (or substantially purified) molecules or may be one component of a mixture of compounds (*e.g.*, an extract or supernatant obtained from cells). In certain embodiments of a mixed compound assay,

expression or activity of an iron-related polynucleotide or polypeptide is tested against progressively smaller subsets of the candidate compound pool until a single compound or minimal compound mixture is demonstrated to modulate expression or activity of an iron-related polynucleotide or polypeptide. Alternatively, diverse mixtures (*i.e.*, libraries) of test compounds may be assayed in such a way that the pattern of response indicates which compounds in the various mixtures are responsible for the effect (deconvolution). Libraries of compounds are commercially available and may be synthesized accordingly to methods known in the art.

In one embodiment, the present invention provides a method of identifying a compound that modulates activity or expression of an iron-related polynucleotide or polypeptide, comprising contacting a cell comprising an iron-related polypeptide or polynucleotide with a compound and determining whether said contacting results in increased expression or activity of an iron-related polypeptide or polynucleotide. The skilled artisan would understand that a variety of different iron-related polypeptides and polynucleotides may be used in such assays, including full length cDNAs and polypeptides, as well as fragments or variants thereof. Accordingly, it is understood that the methods described herein may be adapted to utilize iron-related polypeptide or polynucleotide fragments or variants. The selectivity of a compound that modulates the activity an iron-related polypeptide to its effect on a control polypeptide.

In certain embodiments, the iron-related polypeptide or polynucleotide employed in these various assays may either be free in solution, attached to a solid support, borne on a cell surface or located intracellularly or associated with a portion of a cell. One skilled in the art can, for example, measure the formation of complexes between an iron-related polypeptide and the compound being tested. Alternatively, one skilled in the art can examine the diminution in complex formation between an iron-related polypeptide and its substrate caused by the compound being tested.

In one embodiment, modulators are identified by their ability to bind to an iron-related polynucleotide or polypeptide, or fragment thereof. Binding molecules are particularly useful in modulating (*i.e.*, blocking, inhibiting or stimulating) biological activities of iron-related polypeptides. Accordingly, the present invention provides a method of identifying a modulator of an iron-related polypeptide comprising contacting an iron-related polypeptide with a compound and determining whether the compound specifically binds to the iron-related polypeptide as compared to a control polypeptide. In particular embodiments, at

least two-fold, three-fold, five-fold, or ten-fold more modulator will be bound to the iron-related polypeptide as to an equivalent amount of an unrelated polypeptide.

Routine binding assays suitable for screening candidate molecules and compounds are well known in the art and include, *e.g.*, GST pulldown assays using recombinantly-produced GST-iron related polypeptide fusion polypeptides, affinity chromatography, phage display, immunoprecipitation assays under low stringency conditions suitable for precipitating iron-related polypeptide complexes using antibodies to iron-related polypeptides, ELISA assays, and radioimmunoassays. In other embodiments, modulators are identified by other means of identifying an iron-related polypeptide's binding partner, such as phage display techniques and yeast two-hybrid screening. Such interactions can be further assayed by means including but not limited to fluorescence polarization or scintillation proximity methods.

In certain embodiments, binding molecules are identified or developed using isolated or recombinant iron-related polypeptide, iron-related polypeptide variants, or cells expressing iron-related polypeptides. In particular embodiments, variants have at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to an iron-related polypeptide. The use of recombinant iron-related polypeptides may allow for better specificity (higher relative purity), provide the ability to generate large amounts of receptor material, and can be used in a broad variety of formats (*see* Hodgson, Bio/Technology, 1992, 10, 973-980; each of which is incorporated herein by reference in its entirety).

In certain embodiment, methods of identifying a modulator of an iron-related polypeptide comprise identifying a compound that modulates (*i.e.*, increases or decreases) an activity of an iron-related polypeptide. These methods comprise contacting an iron-related polypeptide with a compound, and determining whether the compound modifies an activity of the iron-related polypeptide. Typically, the iron-related polypeptide will be present in a cell or animal. In this approach, the degree of biological activity of the iron-related polypeptide in the presence of a candidate compound is compared to the degree of activity in its absence, under equivalent conditions. Where the activity of the sample containing the test compound is higher than the activity in the sample lacking the test compound, the compound will have increased activity. Similarly, where the activity of the sample containing the test compound is lower than the activity in the sample lacking the test compound, the compound will have inhibited activity. Biological

activity of an iron-related polypeptide may be measured by any standard assay, for example, those described herein.

Suitable assays for measuring the binding of a modulator to a target protein or for measuring the ability of a modulator to affect a biological activity of a target protein include, but are not limited to, Western blot, immunoblot, enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), immunoprecipitation, surface plasmon resonance, chemiluminescence, fluorescent polarization, phosphorescence, immunohistochemical analysis, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, microcytometry, microarray, microscopy, fluorescence activated cell sorting (FACS), and flow cytometry. Other assays include cell-based assays such as: cytokine secretion assays, or intracellular signal transduction assays that determine, for example, protein or lipid phosphorylation, mediator release or intracellular Ca^{++} mobilization.

EXAMPLES

EXAMPLE 1

VALIDATION OF IRON OVERLOAD MODEL

In order to identify iron-regulated genes, a murine model of iron loading was established. C57BL/6 male mice were overloaded by diet supplementation with carbonyl-iron at various concentrations: 0%, 0.5%, 1.5% and 3%. The duration of iron loading was 1, 2 or 4 months. Following 2 and 4 months iron loading, liver iron content (LIC) was evaluated to determine the degree of iron loading present. As shown in Figure 1, the iron content in the liver plateaued at 2 months, whereas in the spleen, iron content continued to increase over time with greater iron content at four compared to two months iron loading. Histopathologic studies confirmed the liver iron loading and demonstrated similar findings as seen in human genetic hemochromatosis, with iron deposits mainly localized within the hepatocytes of the periportal area (Figure 2). Thus, carbonyl-iron loaded C57BL/6 male mice represented a good model for adult onset hemochromatosis (HH).

In order to fully validate the established iron overload model, mRNA expression of liver-related genes known to be involved in iron metabolism was characterized using RealTime-PCR (Table 2). The impact of iron load was analyzed by comparing 0% and 3% animals at the various times. Statistical differences are indicated with arrows ($p<0.05$).

Table 2. Iron responsive genes

			Impact of Iron Treatment
Iron Parameter	Liver iron concentration		↑
	Spleen iron concentration		↑
	Plasma iron		↑
	TIBC		=
	Transferrin saturation		↑
	NTBI		↑
	Hemoglobin		=
Hepatic mRNA Expression Level	Transport	Transferrin	=
	Uptake	Tfr1	↓
		Dmt1	=
		Dcytb	=
	Storage	H-ferritin	=
		L-ferritin	↑
	Release	Ferroportin	↑
		Ceruloplasmin	=
	Iron regulators	Hepcidin1	↑
		Hfe	=
		Tfr2	=
		Hjv	=

"=" represents genes not significantly modulated
TIBC = Total iron binding capacity
NTBI = Non transferring bound iron
Tfr = Transferrin receptor

Using this strategy, two sets of iron metabolism-related genes were identified: those that are iron-responsive and those that are not. The iron-responsive group included hepcidin1, ferroportin, L-ferritin, and transferrin receptor 1 (Tfr1), while the relatively non-responsive genes included HFE, hemojuvelin, transferrin receptor 2 (Tfr2), ceruloplasmin, Dcytb, DMT1 and H-ferritin. The relationship between the levels of gene expression and serum iron parameters at different times was analyzed (data not shown). These results showed that this model is behaving in a manner consistent with existing knowledge of iron metabolism and regulation. For example, as expected, transferrin receptor 1 (Tfr1) mRNA levels were inversely correlated with iron load, whereas hepcidin levels were directly correlated with iron load. Thus, based on biochemical,

histopathological and gene expression data, this model of oral iron loading was validated for further studying iron overload.

EXAMPLE 2

IDENTIFICATION OF IRON MODULATED GENES

Using the animal model described in Example 1, expression array profiling comparing the 2-months, 3% carbonyl iron supplementation group to the no-loading (0%) group was performed, using RNA-based microarray technology to identify panels of genes differentially regulated in liver and spleen during iron loading. A non-biased exploratory microarray approach comprising a pangenomic chip representing 30,000 genes was initially used. Experiments were performed using two different pools of mice (3 in each pool), and a dye-swap hybridization was used to exclude a dye specific effect on gene expression.

A summary of the differentially expressed genes identified is provided in Table 3. These genes exhibited a modulation of the mRNA expression in C57BL/6 mice either in the liver or in spleen. Most of the genes had not been previously associated with iron metabolism. Statistical Analysis Microarray method (SAM)[6] was used to identify differentially expressed genes, which were subjected to RT-PCR analyses to confirm altered expression profiles in three different iron-related models (Table 3). The first model was a carbonyl-iron oral loading murine model corresponding to mice overloaded for 2 months as described above. The second model was an iron dextran model corresponding to mice injected once with 1mg/kg of iron-dextran and sacrificed two months later. This model displayed macrophage iron overload and circumvented the possibility that altered gene expression was related to the digestive absorption process. The third model was an acute anemia model corresponding to mice in which 3 blood withdrawals were performed within the previous three days. Those genes that showed the expected response in all the models were identified. For example, gene LTF showed a 2-4 fold induction with iron loading in both the oral and the parenteral loading models of iron overload, and a 3-fold reduction in the iron deficient anemia model.

Table 3: Genes differentially-regulated in iron loading

			DNA chip Modulation	Differential level by qPCR		
Murine Symbol	Human symbol	Organ	Carbonyl-Iron		Iron Dextran	Acute Anemia
Apoa4	APOA4	Liver	↑	3	5	NM
Onecut1	ONECUT1			5	NM	NM
Id2	ID2			2	2	NM
Mt1	MT1A			4	NM	NM
Tgfb1i4	TSC22D1		↓	-3	-6	NM
2610510B01	C21orf5			-5	-2	NM
Vegfb	VEGFB			-2	NM	2
Ugt3a2	FLJ34658			-1,5	NM	NM
Vnn1	VNN1			-5	NM	NM
Col27a1	COL27A1			-3	NM	NM
Pparg	PPARG			-5	NM	NM
Ugt3a1	UGT3A2			-3,5	NM	NM
Hsd17b9	HSD17B6			-5	-2	NM
Mmp8	MMP8	Spleen	↑	5	2	-4
Ltf	LTF			4	2	-3
Dyrk3	DYRK3			4	NM	NM
2810417H13	KIAA0101			2	NM	NM
Car2	CA2			2	NM	NM
Chek1	CHEK1			2,5	NM	NM
Chi3l3				4	2	NM
Hspca	HSPCA			2,5	NM	NM
Spna1	SPTA1			5	2	NM
Aurka	STK6			4	2	NM
D6Wsu116e	FAM21C		↓	-1,5	NM	NM

NM: not modulated

The genes derived from these studies were subjected to bioinformatic analyses and literature review to identify a subset of genes having corresponding proteins that are secreted into the plasma. Table 4 provides a list of these secreted proteins, as well as other secreted proteins implicated in iron metabolism, iron homeostasis, or iron-related disorders.

Table 4. Secreted proteins

Hepcidin	Haptoglobin	Ceruloplasmin
Hemojuvelin	VNN1	APOA4
Transferrin	Erythropoietin	Catalase
Transferrin receptor 1	IL-6	CD163
Ferritin H	TNF a	Hemopexin
Ferritin L	MMP8	Lactoferrin
Heme oxygenase-1	BMP2	VEGFB

These secreted proteins represent useful targets for diagnostic and prognostic applications related to iron-related disorders, and compounds that bind a subset or all of these proteins may be included in a proteomic panel for detecting levels of these secreted proteins.

EXAMPLE 3

CHARACTERIZATION OF IRON-REGULATED TSC22 EXPRESSION

The TSC22D1 gene (TSC22), which encodes a transcriptional factor, was previously associated with the BMP and TGF beta pathways, both of which are involved in the control of iron metabolism. To further elucidate the role of TSC22 in iron metabolism, its expression was analyzed in various models of iron overload or deficiency by RT-PCR using primers that specifically amplified the TSC22 gene.

1. Hepatic TSC22D1 transcript level and protein are down-regulated in iron overload mice (Figure 3)

The expression levels of TSC22 during iron overload were determined using two different carbonyl-iron mice models. The first model was the B57BL/6 mice strain described in Example 1, and the second model was Balb/c mice. Iron overload was performed by diet supplementation with carbonyl iron at various doses for 1, 2, and 4 months. Iron parameters were significantly different in the Balb/c mice as compared to those observed in the C57BL/6 strain, especially in iron overloaded mice. Biochemical and histological analysis confirmed the development of the iron overload in the liver and in the spleen. In the liver of B57BL/6 mice, hepatocytes were the only cells presenting significant amounts of excess iron deposits. However, in the Balb/c mice, iron deposits were

also found in Kupffer cells. Schematically, the iron overload was most similar to the observations made during GH in C57BL/6 and during inflammatory process in BALB/c. Such findings are associated with strong differences in the interrelationships between the hepatic mRNA levels of the iron metabolism genes. The C57BL/6 mice strain was used in a first approach. Biochemical and histological analysis confirmed the development of the iron overload in the liver and in the spleen. In the liver, hepatocytes were the only cells presenting significant amount of iron deposits in excess.

In both carbonyl-iron mice models, the hepatic Tsc22d1 mRNA level was significantly down-regulated in carbonyl iron overloaded compared to control mice at 2 or 4 months of treatment. This decrease of mRNA level was associated with a decrease of the corresponding TSC22D1 protein level in C57BL/6 mice. The decrease of TS22D1 expression was also observed in iron-dextran mice models.

2. Hepatic TSC22D1 transcript level and protein are up-regulated in iron deficiency mice (Figure 4)

In mice maintained in an iron deficient diet, liver iron concentration and haemoglobin levels were not affected, but the hepatic TSC22D1 mRNA expression level was significantly increased at 30 days compared to control mice. In the same time, the hepcidin expression level was significantly decreased.

3. Hepatic TSC22 transcript level and protein were not modulated during acute anemia (Figure 5)

TSC22D1 expression level during acute anemia was determined by performing blood punction in C57bL/6 male mice during the 45 hours prior to obtaining an mRNA sample for RT-PCR. The TSC22D1 mRNA expression level was not modified in the liver.

4. TSC22 transcript level are modulated in iron-overload cells (Figure 6)

To analyse cellular expression of Tcs22d1 mRNA in the liver, hepatocytes and non parenchymal cells were obtained after liver dissociation of iron dextran overloaded mice and control dextran injected mice. One week after the injection of iron-dextran in C57bl/6 male mice, iron was found preferentially accumulated in Kupffer cells. At this time, Tsc22d1 mRNA expression level in iron-overloaded, non parenchymal cells was significantly decreased compared to the

same cells in control-dextran liver. In hepatocytes, Tsc22d1 expression level did not vary 1 week after the injection. Two months after the iron-dextran injection, iron was found preferentially accumulated in hepatocytes. Tsc22d1 mRNA expression was significantly decreased in hepatocytes and was not modified in non parenchymal cells. These results demonstrate that Tsc22d1 mRNA expression levels vary with iron content in hepatocytes and non paremchymal cells enriched populations.

5. TSC-22 transcript levels are modulated by iron in Huh7 cells (Figure 7)

Human Hepatoma cells lines, HuH7 cells, were used to study, *in vitro*, the modification of TSC22D1 mRNA expression level when iron status was modified. HuH7 cells were maintained in iron-citrate medium for 48 hours. Under this condition, TFRC mRNA expression was significantly decreased compared to its expression in cells maintained in control citrate medium. The HAMP mRNA expression level was also significantly decreased. TSC22D1 mRNA expression level was significantly decreased compared to its level in HuH7 cells maintained in control-citrate medium. In opposing conditions, when HuH7 cells were maintained in the presence of Deferoxamine (DFO) for 48 hours, the TFRC mRNA expression level was significantly increased, without modification of the Hamp mRNA expression level, as compared to their expression levels in cells maintained in control medium. Under this condition, the TSC22D1 mRNA expression level was also significantly increased.

6. Inhibition of TSC-22 transcript level is associated with decrease of TFRC expression level without modification of cellular iron content (Figure 8)

To determine the impact of TSC22D1 on cellular iron metabolism, TSC22D1 expression was inhibited in HuH7 cells using specific siRNA. 72 hours later, TFRC mRNA expression level was significantly decreased by TSC22D1 mRNA inhibition. This decrease of TFRC mRNA was not associated to a modulation of Hamp1 mRNA expression level. In addition, the inhibition of the TSC22D1 expression in HuH7 cells produced a slightly significant decrease of the IRE form of Dmt1 mRNA. However, no modulation of the cellular ferritin protein level was observed.

7. Effect of TSC-22 over-expression in HuH7 cells (Figure 9)

A preliminary study was performed to determine the effect of TSC22 overexpression in HuH7 cells. A TSC-22 expression vector or control plasmid was transfected into HuH7 cells. Twenty-four hours after the transfection in HuH7 cells, the TSC-22 mRNA expression level was increased compared to the level in HuH7 cells transfected with control plasmid. Overexpression of TSC-22 was associated with an increase of TFRC mRNA expression without modulation of hepcidin mRNA expression.

EXAMPLE 4

CHARACTERIZATION OF IRON –REGULATED LACTOFERRIN GENE EXPRESSION (FIGURE 9)

The lactoferrin gene encodes a secreted protein associated with iron regulation. Its biological activities include an antimicrobial activity due to iron deprivation resulting of lactoferrin secretion in extracellular medium.

Plasma concentrations of lactoferrin were determined in various situations, including genetic hemochromatosis, cirrhosis, and hepatosiderosis, by using an ELISA test. Lactoferrin concentration in plasma was increased in all of these situations.

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

1. A method for determining whether a patient has an iron-related disorder, said method comprising measuring in a patient or in a biological sample obtained from said patient the level of expression of one or more polynucleotides comprising a sequence set forth in any one of SEQ ID NOs: 111, 98-110, and 112-178, or a homolog or ortholog thereof, wherein an altered level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder.

2. The method of claim 1, comprising measuring the level of expression of two or more polynucleotides.

3. The method of claim 1, wherein said polynucleotides are mRNAs.

4. The method of claim 1, wherein the level of expression of said one or more polynucleotides is greater than the normal control level.

5. The method of claim 1, wherein the level of expression of said one or more polynucleotides is less than the normal control level.

6. A method for determining whether a patient has an iron-related disorder, said method comprising determining the presence or absence of a mutation or polymorphism in one or more of a patient's genes encoding polypeptides having a sequence set forth in any one of SEQ ID NOs: 190, 179-189, and 191-253, or a homolog or ortholog thereof, wherein the presence of said mutation or polymorphism indicates that said patient has an iron-related disorder.

7. A method for determining whether a patient has an iron-related disorder, said method comprising measuring in said patient or a biological sample obtained from said patient the level of biological activity of one or more polypeptides having an amino acid sequence set forth in any one of SEQ ID NOs: 190, 179-189, and 191-253, or a homolog or ortholog thereof, wherein an altered level in said biological activity, relative to a normal control level, indicates that said patient has an iron-related disorder.

8. A method for determining whether a patient has an iron-related disorder, said method comprising measuring in said patient or a biological sample obtained from said patient the level of expression of one or more polypeptides having an amino acid sequence set forth in any one of SEQ ID NOs: 190, 179-189, and 191-253, or a homolog or ortholog thereof, wherein an altered level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder.

9. A method for determining whether a patient has an iron-related disorder related to iron overload, said method comprising measuring the level of expression of one or more polynucleotides comprising a sequence set forth in any one of SEQ ID NOs: 98-110, 124-140, 148, 149, and 166, or a homolog or ortholog thereof, or of one or more polypeptides comprising a sequence set forth in any one of SEQ ID NOs: 179-189, 203-215, 223, 224 and 241, or a homolog or ortholog thereof, wherein an increased level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder related to iron overload.

10. A method for determining whether a patient has an iron-related disorder related to iron deficiency, said method comprising measuring the level of expression of one or more polynucleotides comprising a sequence set forth in any one of SEQ ID NOs: 98-110, 124-140, 148, 149, and 166, or a homolog or ortholog thereof, or of one or more polypeptides comprising a sequence set forth in any one of SEQ ID NOs: 179-189, 203-215, 223, 224 and 241, or a homolog or ortholog thereof, wherein a decreased level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder related to iron overload.

11. A method for determining whether a patient has an iron-related disorder related to iron overload, said method comprising measuring the level of expression of one or more polynucleotides comprising a sequence set forth in any one of SEQ ID NOs: 111-123, 143, and 151, or a homolog or ortholog thereof, or of one or more polypeptides comprising a sequence set forth in any one of SEQ ID NOs: 190-202, 216-218, and 226, or a homolog or ortholog thereof, wherein a decreased level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder related to iron overload.

12. A method for determining whether a patient has an iron-related disorder related to iron deficiency, said method comprising measuring the level of expression of one or more polynucleotides comprising a sequence set forth in any one of SEQ ID NOs: 111-123, 143, and 151, or a homolog or ortholog thereof, or of one or more polypeptides comprising a sequence set forth in any one of SEQ ID NOs: 190-202, 216-218, and 226, or a homolog or ortholog thereof, wherein an increased level in said expression, relative to a normal control level, indicates that said patient has an iron-related disorder related to iron deficiency.

13. A method for determining if a treatment for an iron-related disorder is efficacious, comprising:

(a) measuring in a patient or in a biological sample obtained from said patient, at a first time point prior to treatment, the level of expression or activity of one or more polynucleotides comprising a sequence set forth in any one of SEQ ID NOs: 111, 98-110, and 112-178, or a homolog or ortholog thereof, wherein said level of expression is altered by a first amount relative to a normal control level; and

(b) measuring in a patient or in a biological sample obtained from said patient, at a second time point following treatment, the level of expression or activity of the one or more polypeptides of step (a), wherein said level of expression is altered by a second amount relative to a normal control level,

wherein if the first amount is greater than the second amount, it indicates that the treatment is efficacious.

14. A method for monitoring the progression of an iron-related disorder in a patient, comprising:

(a) measuring in a patient diagnosed with or suspected of having an iron-related disorder, or in a biological sample obtained from said patient, at a first time point, a level of expression or activity of one or more polynucleotides comprising a sequence set forth in any one of SEQ ID NOs: 111, 98-110, and 112-178, or a homolog or ortholog thereof, and determining a first relative value by which the level is altered as compared to a normal control level;

(b) measuring in the patient or in a biological sample obtained from said patient, at a later second time point, a level of expression or activity of the one or more polypeptides of step (a) and determining a second relative value by which the level is altered as compared to a normal control level,

wherein if the first relative value is less than the second relative value, it indicates that the iron-related disorder is progressing, and if the first relative value is greater than the second relative value, it indicates that the iron-related disorder is in remission.

15. A method for identifying a compound for the treatment or prevention of an iron-related disorder, said method comprising:

(a) contacting a cell expressing a polynucleotide encoding a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 180, 179-189, and 191-253, or a variant or fragment thereof, with a candidate compound; and

(b) measuring the level of expression or a biological activity of said polynucleotide or polypeptide,

wherein an altered level of expression or biological activity of said polynucleotide or polypeptide, relative to the level in a cell not contacted with said compound, indicates that said candidate compound is useful for the treatment of an iron-related disorder.

16. A method for identifying a compound for the treatment or prevention of an iron-related disorder, said method comprising contacting a polypeptide having an amino acid sequence set forth in any one of SEQ ID Nos: 190, 49-97, 179-189, and 191-253, or a variant or fragment thereof, with a candidate compound; and determining whether said candidate compound binds to said polypeptide, wherein binding of said candidate compound to said polypeptide identifies said candidate compound as a compound useful for the treatment or prevention of an iron-related disorder.

17. A method for identifying a compound for the treatment of an iron-related disorder, said method comprising administering a candidate compound to a transgenic animal expressing a transgene encoding a polypeptide having an amino acid sequence set forth in any one of SEQ ID Nos: 190, 49-97, 179-189, and 191-253, or a variant or fragment thereof, and determining whether said candidate compound alters a biological activity or the level of expression of said transgene or polypeptide, wherein an alteration in the biological activity or level of expression of said transgene or polypeptide identifies said candidate compound as a compound useful for the treatment of an iron-related disorder.

18. A method for identifying a compound for the treatment of a iron-related disorder, said method comprising the steps of administering a candidate compound to a transgenic animal expressing a transgene encoding a polypeptide having an amino acid sequence set forth in any one of SEQ ID Nos: 190, 49-97, 179-189, and 191-253, or a variant or fragment thereof, said animal having an iron-related disorder; and determining whether said candidate compound alleviates said iron-related disorder.

19. A method for identifying a compound for the treatment of an iron-related disorder, said method comprising the steps of administering a candidate compound to a animal comprising a mutation in a gene encoding a polypeptide having an amino acid sequence set forth in any one of SEQ ID Nos: 190, 49-97, 179-189, and 191-253, or an ortholog thereof, and determining whether said candidate compound alters a biological activity or the level of expression of said gene or polypeptide, wherein an alteration in the biological activity or level of expression of said polypeptide identifies said candidate compound as a compound useful for the treatment of an iron-related disorder.

20. A method for identifying a compound for the treatment of an iron-related disorder, said method comprising the steps of administering a candidate compound to a animal comprising a mutation in a gene encoding a polypeptide having an amino acid sequence set forth in any one of SEQ ID Nos: 190, 49-97, 179-189, and 191-253, or an ortholog thereof, said animal having an iron-related disorder; and determining whether said candidate compound alleviates said iron-related disorder.

21. An animal model of an iron-related disorder, wherein said animal model is a non-human mammal comprising a transgene that expresses a polypeptide having an amino acid sequence set forth in any one of SEQ ID Nos: 190, 49-97, 179-189, and 191-253, or a fragment or variant thereof, wherein said non-human mammal exhibits one or more symptoms of an iron-related disorder.

22. An animal model of an iron-related disorder, wherein said animal model is a non-human mammal comprising a mutation in a gene encoding a polypeptide having an amino acid sequence set forth in any one of SEQ ID Nos: 190, 49-97, 179-189, and 191-253, or an ortholog thereof, wherein said non-human mammal exhibits one or more symptoms of a iron-related disorder.

23. A cell isolated from the non-human mammal of claim 21 or claim 16.

24. A kit for determining the presence of an iron-related disorder in a patient, wherein said kit comprises one or more compound that specifically bind to a polypeptide having a sequence set forth in any one of SEQ ID NOs: 190, 179-189, and 191-253 or specifically bind to a polynucleotide that encodes a polypeptide having a sequence set forth in any one of SEQ ID NOs: 190, 179-189, and 191-253.

25. The kit of claim 24, wherein said kit comprises an array of compounds that specifically bind to polypeptides having a sequence set forth in any one of SEQ ID NOs: 190, 179-189, and 191-253 or specifically bind to polynucleotides that encode polypeptides having a sequence set forth in any one of SEQ ID NOs: 190, 179-189, and 191-253.

26. The kit of claim 24 or 25, wherein said one or more compounds are labeled.

27. The kit of claim 24 or 25, wherein said compounds are oligonucleotides.

28. The kit of claim 24 or 25, wherein said polypeptides are secreted.

29. A method of treating or preventing an iron-related disorder in a patient, said method comprising administering to said patient a nucleic acid molecule encoding a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 190, 49-97, 179-189, and 191-253, or a variant or fragment thereof.

30. The method of claim 29, wherein said method comprises administering to said patient an expression vector comprising a nucleic acid molecule operably linked to a promoter, said nucleic acid molecule encodes a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 190, 49-97, 179-189, and 191-253, or a variant or fragment thereof.

31. A method of treating or preventing an iron-related disorder in a patient, said method comprising administering to said patient a compound that modulates a biological activity or expression of a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 190, 179-189, and 191-253.

32. The method of claim 31, wherein said compound is a nucleic acid molecule comprising a portion of a polynucleotide sequence set forth in any one of SEQ ID NOs: 111, 1-48, 98-110, and 112-178 or a complement thereof.

33. The method of claim 32, wherein said nucleic acid molecule is selected from the group consisting of: viruses, plasmids, antisense RNA, ribozymes, and RNAi oligonucleotides.

34. The method of claim 31, wherein said compound is an antibody that specifically binds a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 190, 49-97, 179-189, and 191-253.

35. The method of claim 31, wherein said compound is a small molecule.

36. The method of claim 31, wherein said compound increases the biological activity or expression of a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 190, 49-97, 179-189, and 191-253.

37. The method of claim 31, wherein said compound decreases the biological activity or expression of a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 190, 49-97, 179-189, and 191-253.

38. A plurality of polynucleotides consisting essentially of polynucleotides comprising a portion of a sequence set forth in any one of SEQ ID NOs: 111, 1-48, 98-110, and 112-178 or a complement thereof.

39. The plurality of polynucleotides of claim 38, wherein said polynucleotides are single-stranded oligonucleotides.

40. The plurality of polynucleotides of claim 38, wherein said polynucleotides are labeled.

41. A plurality of antibodies consisting essentially of antibodies that specifically bind polypeptides having a sequence set forth in any one of SEQ ID NOs: 190, 49-97, 179-189, and 191-253.

42. The plurality of antibodies of claim 41, wherein said antibodies are monoclonal antibodies.

43. The plurality of antibodies of claim 41, wherein said antibodies are labeled.

44. The plurality of antibodies of claim 41, wherein said polypeptides are secreted.

45. The method of any one of claims 1, 11-14, and 32, wherein the polynucleotide comprises a sequence set forth in any one of SEQ ID NOs: 111 and 112.

46. The method of any one of claims 6-8, 11, 12, 15-20, 29-31, and 34-37 37, wherein the polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 190 and 191.

47. The animal model of any one of claims 21 and 22, wherein the polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 190 and 191.

48. The cell of claim 23, wherein the polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 190 and 191.

49. The kit of any one of claims 24-28, wherein the polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 190 and 191.

50. The plurality of polynucleotides of any one of claims 38-40, wherein said plurality comprises a polynucleotide comprising a portion of a sequence set forth in any one of SEQ ID NOs: 111 and 112.

51. The plurality of antibodies of any one of claims 41-44, wherein said plurality comprises an antibody that specifically binds a polypeptide having a sequence set forth in any one of SEQ ID NOs: 190 and 191.

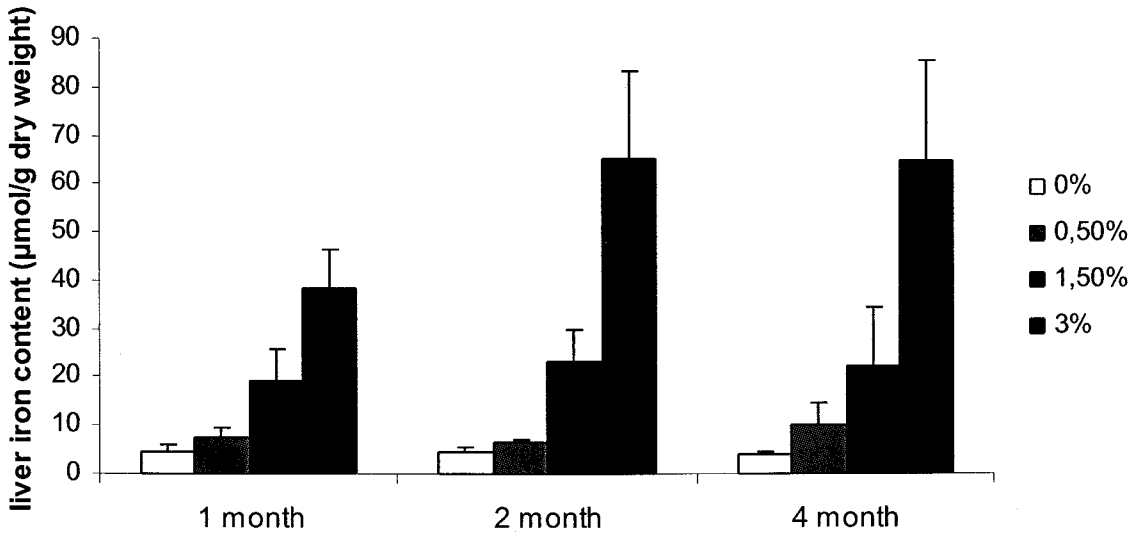

liver iron content (µmol/g dry weight)
90
80
70
60
50
40
30
20
10
0
1 month
2 month
4 month
0%
0,50%
1,50%
3%

FIG. 1A

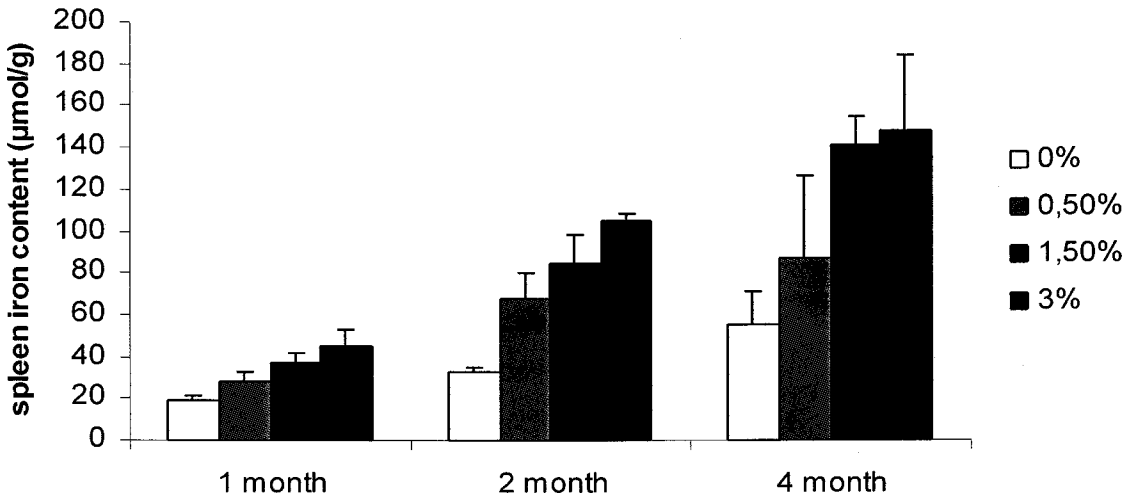

spleen iron content (µmol/g)
200
180
160
140
120
100
80
60
40
20
0
1 month
2 month
4 month
0%
0,50%
1,50%
3%

FIG. 1B

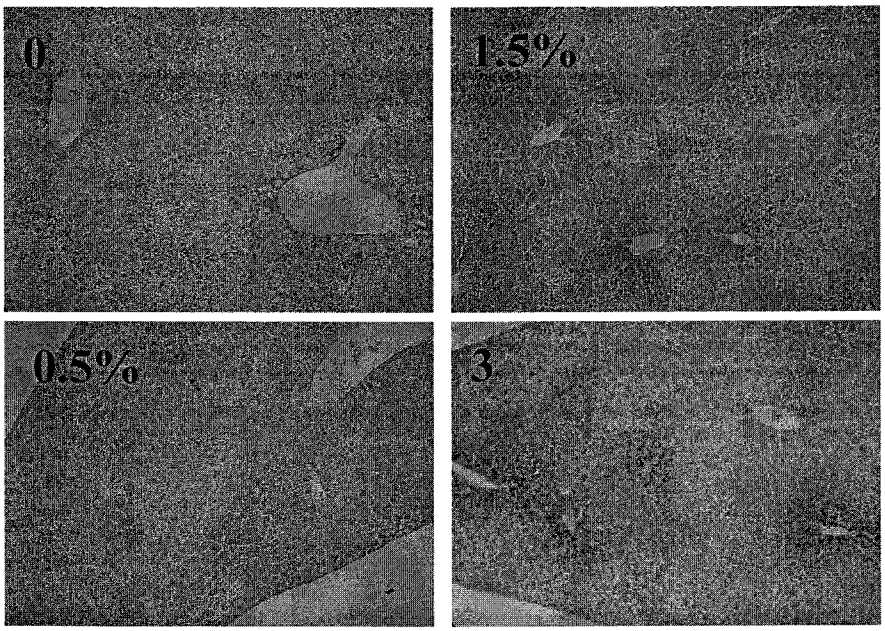


FIG. 2

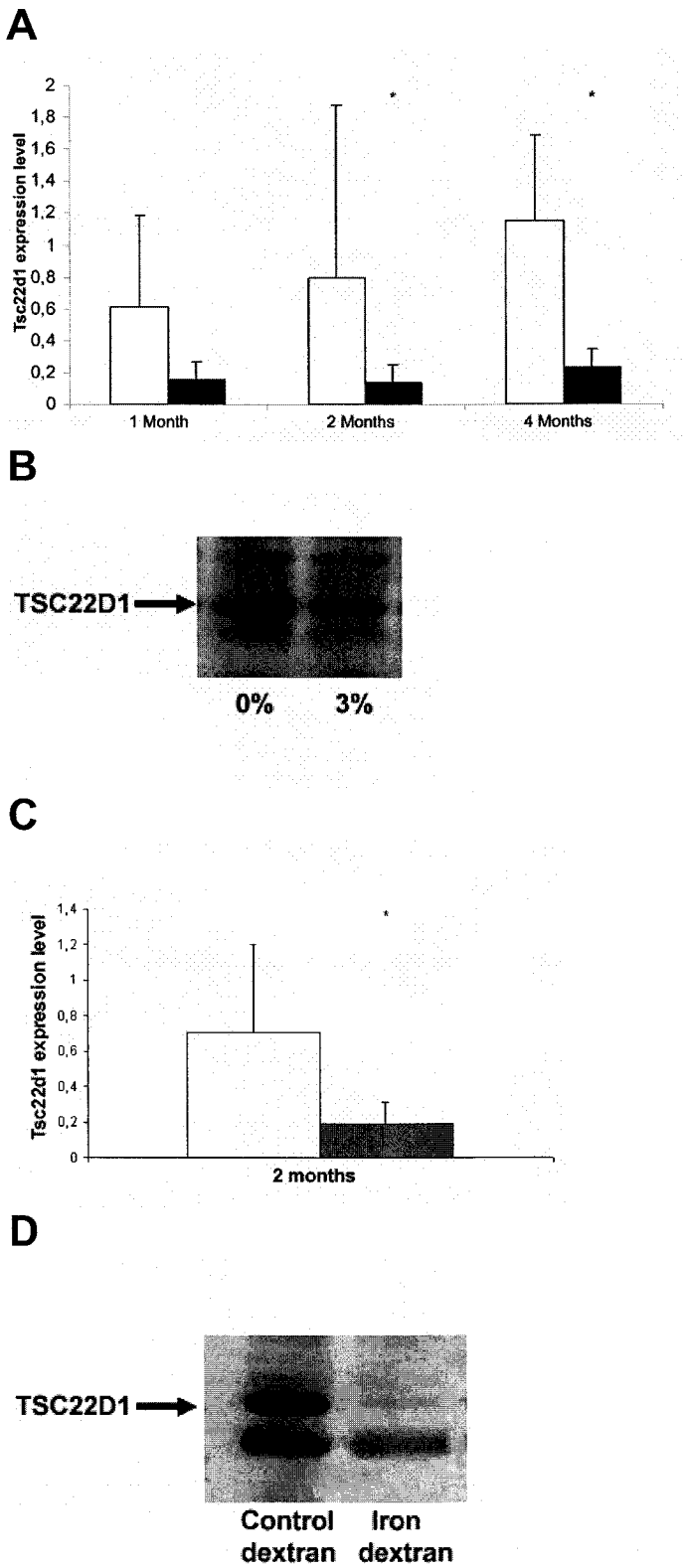

A
Tsc22d1 expression level
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1 Month
2 Months
4 Months
B
TSC22D1
0%
3%
C
Tsc22d1 expression level
1,4
1,2
1
0,8
0,6
0,4
0,2
0
2 months
D
TSC22D1
Control dextran
Iron dextran

FIG. 3

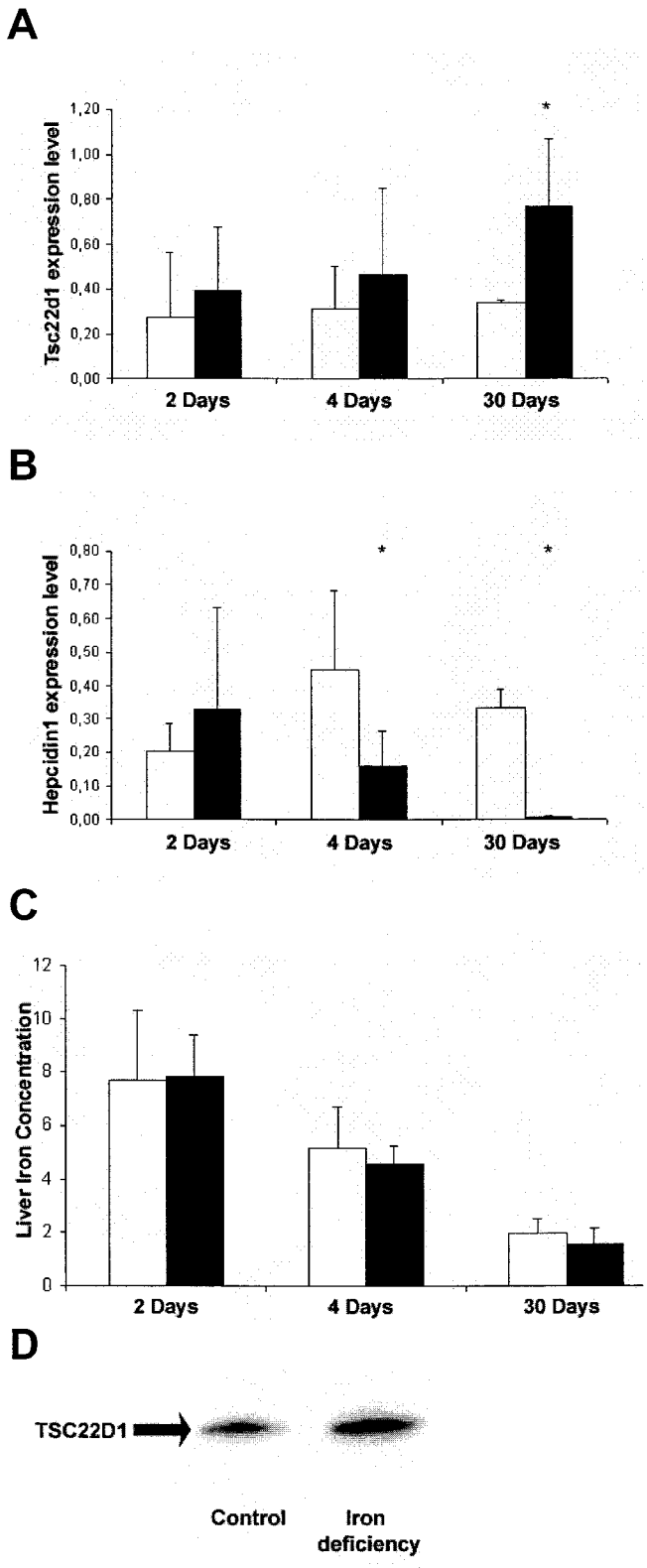

A
Tsc22d1 expression level
1,20
1,00
0,80
0,60
0,40
0,20
0,00
*
2 Days
4 Days
30 Days
B
Hepcidin1 expression level
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
*
*
2 Days
4 Days
30 Days
C
Liver Iron Concentration
12
10
8
6
4
2
0
2 Days
4 Days
30 Days
D
TSC22D1
Control
Iron deficiency

FIG. 4

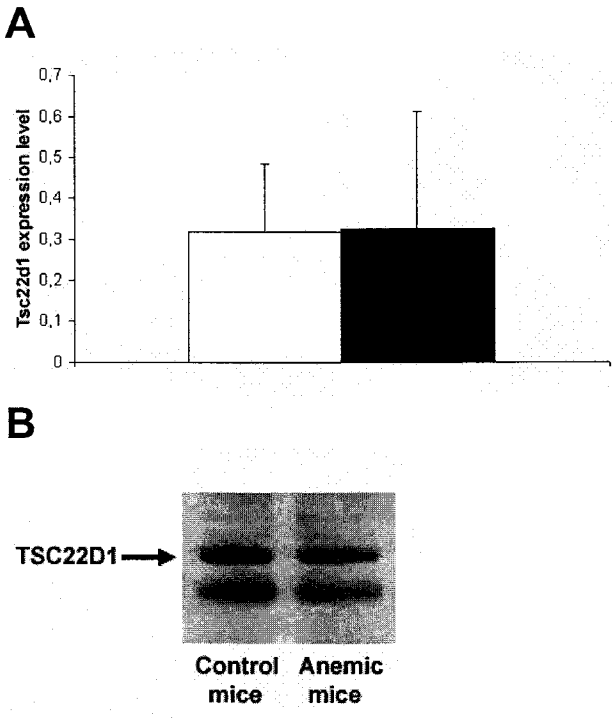
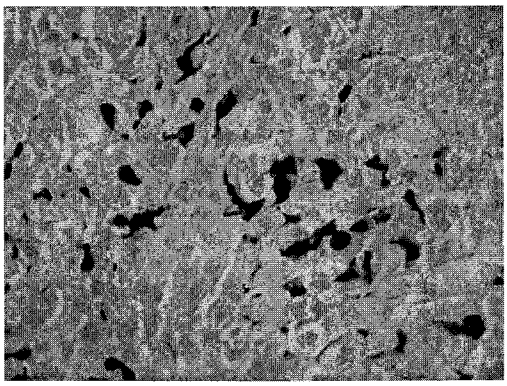
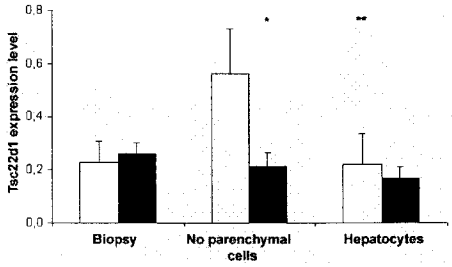

A
Tsc22d1 expression level
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
B
TSC22D1
Control mice
Anemic mice

FIG. 5

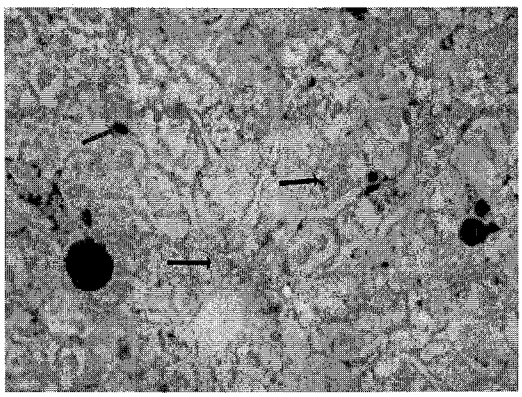
A



B


Tsc22d1 expression level
0,8
0,6
0,4
0,2
0,0
*
**
Biopsy
No parenchymal cells
Hepatocytes

C



D

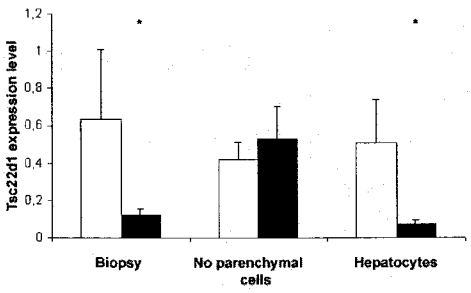

Tsc22d1 expression level
1,2
1
0,8
0,6
0,4
0,2
0
*
*
Biopsy
No parenchymal cells
Hepatocytes

FIG. 6

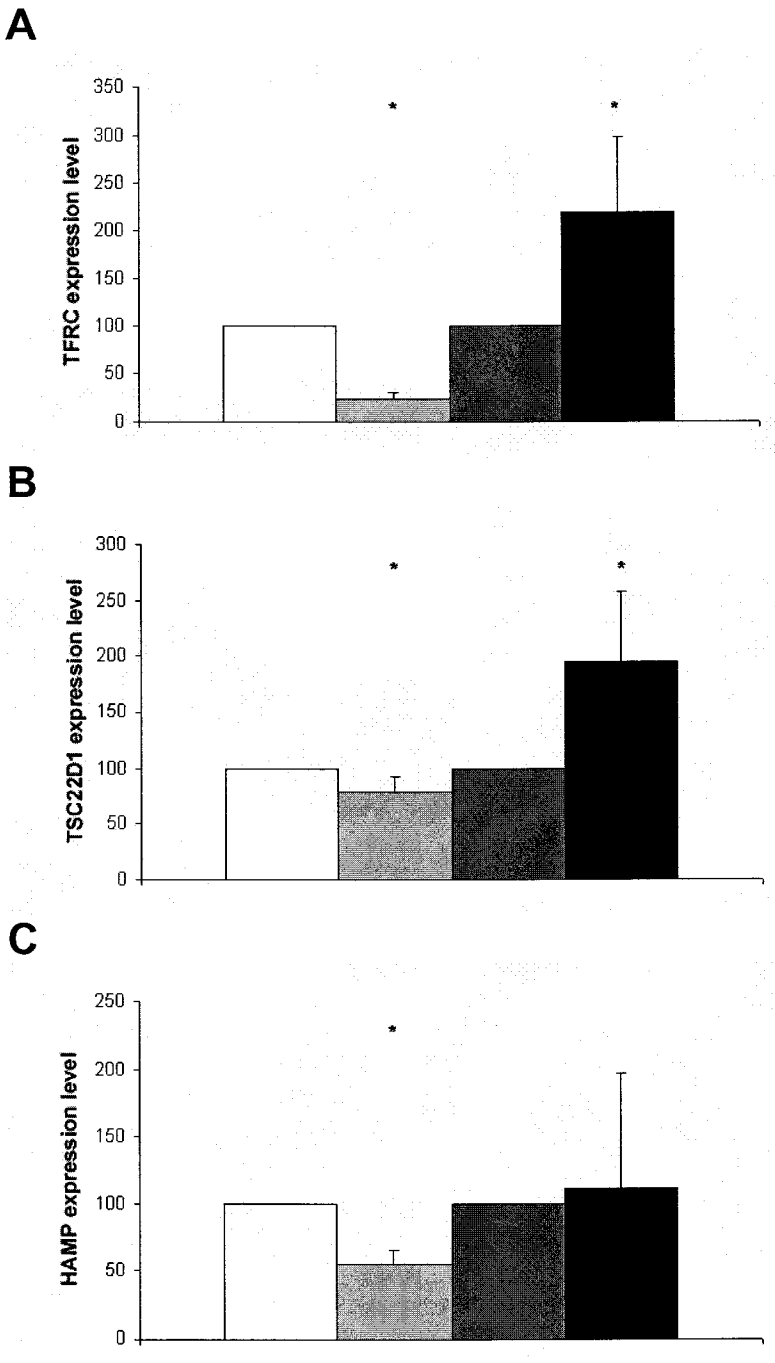

A
TFRC expression level
350
300
250
200
150
100
50
0
*
*
B
TSC22D1 expression level
300
250
200
150
100
50
0
*
*
C
HAMP expression level
250
200
150
100
50
0
*

FIG. 7

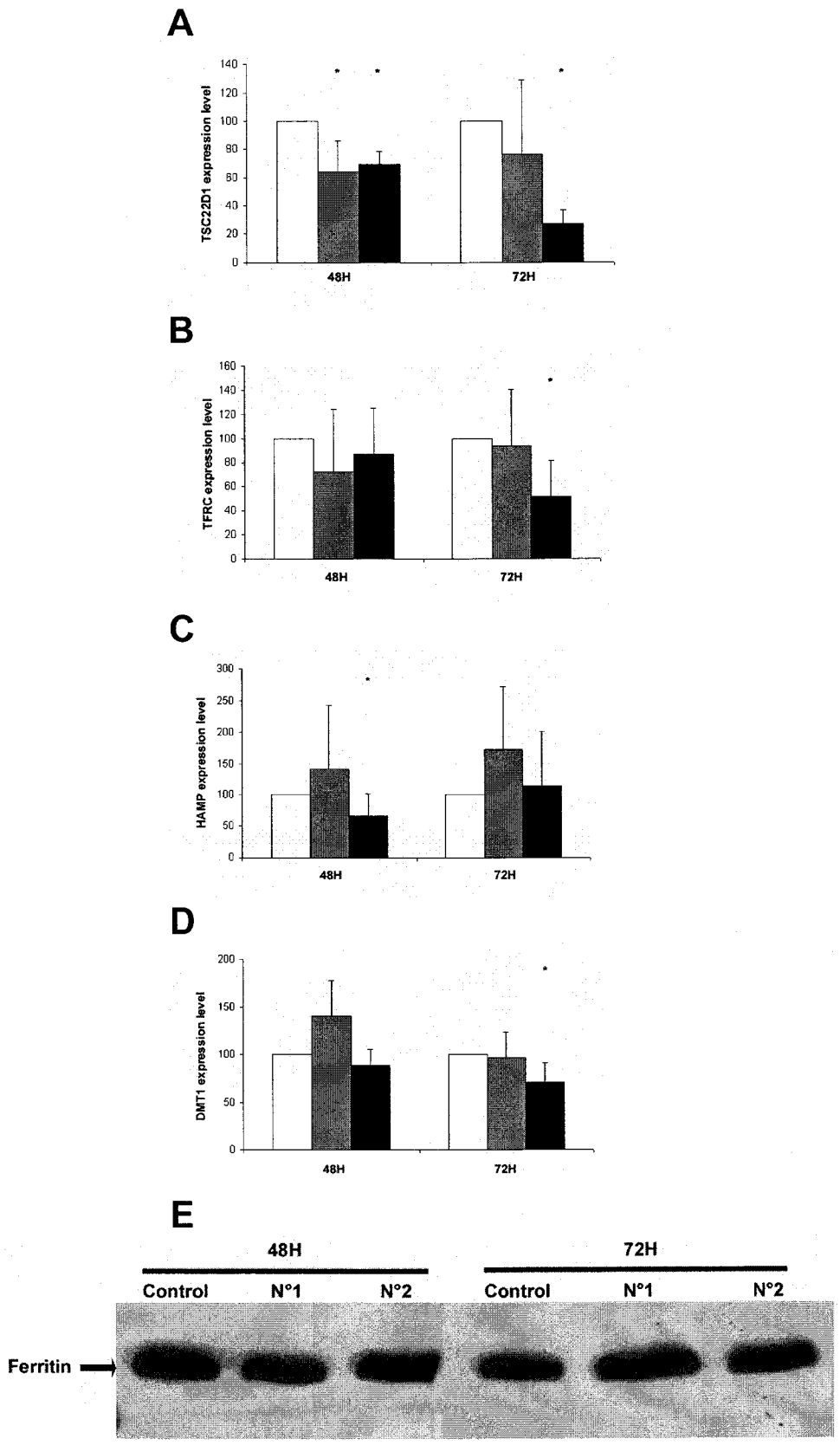

A
TSC22D1 expression level
140
120
100
80
60
40
20
0
48H
72H
B
TFRC expression level
160
140
120
100
80
60
40
20
0
48H
72H
C
HAMP expression level
300
250
200
150
100
50
0
48H
72H
D
DMT1 expression level
200
150
100
50
0
48H
72H
E
48H
72H
Control
N°1
N°2
Control
N°1
N°2
Ferritin

Fig. 8

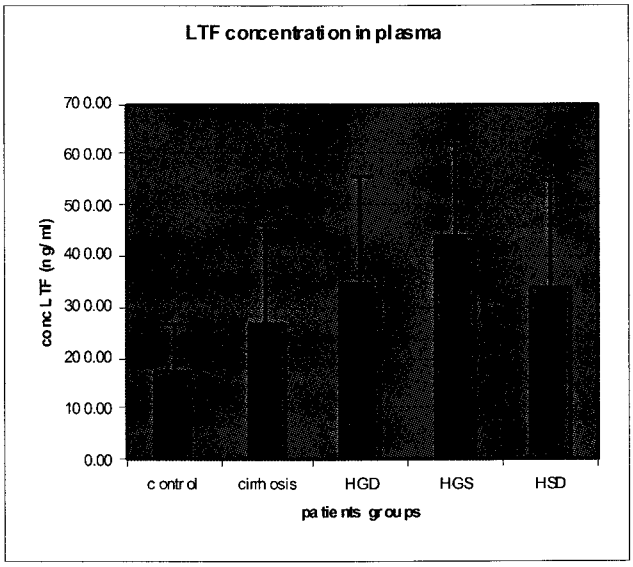

LTF concentration in plasma
70 0.00
60 0.00
50 0.00
40 0.00
30 0.00
20 0.00
10 0.00
0.00
conc LTF (n g/ ml)
control
cirrhosis
HGD
HGS
HSD
patients groups

FIG. 9