nature medicine

High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin

Toshihiko Tanno[1], Natarajan V Bhanu[1], Patricia A Oneal[1], Sung-Ho Goh[1], Pamela Staker[1], Y Terry Lee[1], John W Moroney[2], Christopher H Reed[2], Naomi LC Luban[3], Rui-Hong Wang[4], Thomas E Eling[5], Richard Childs[6], Tomas Ganz[7], Susan F Leitman[8], Suthat Fucharoen[9] & Jeffery L Miller[1]

In thalassemia, deficient globin-chain production during erythropoiesis results in anemia[1–3]. Thalassemia may be further complicated by iron overload (frequently exacerbated by blood transfusion), which induces numerous endocrine diseases, hepatic cirrhosis, cardiac failure and even death[4]. Accumulation of iron in the absence of blood transfusions may result from inappropriate suppression of the iron-regulating peptide hepcidin by an erythropoietic mechanism[5]. To test this hypothesis, we examined erythroblast transcriptome profiles from 15 healthy, nonthalassemic donors. Growth differentiation factor 15 (GDF15), a member of the transforming growth factor-β superfamily, showed increased expression and secretion during erythroblast maturation. Healthy volunteers had mean GDF15 serum concentrations of 450 ± 50 pg/ml. In comparison, individuals with β-thalassemia syndromes had elevated GDF15 serum levels (mean 66,000 ± 9,600 pg/ml; range 4,800–248,000 pg/ml; $P < 0.05$) that were positively correlated with the levels of soluble transferrin receptor, erythropoietin and ferritin. Serum from thalassemia patients suppressed hepcidin mRNA expression in primary human hepatocytes, and depletion of GDF15 reversed hepcidin suppression. These results suggest that GDF15 overexpression arising from an expanded erythroid compartment contributes to iron overload in thalassemia syndromes by inhibiting hepcidin expression.

Because humans have no physiological means for excreting iron, hepcidin-regulated intestinal absorption represents a principal mechanism for iron homeostasis in humans[6]. Hepcidin is a small peptide produced in the liver that reduces iron absorption by causing the degradation of the enterocyte iron transporter ferroportin[7]. Blood transfusion induces iron loading in many patients with thalassemia; if hepcidin expression were regulated predominantly by iron, hepcidin expression should be increased in these individuals. However, it was recently shown that hepcidin levels are decreased in individuals with β-thalassemia syndromes[8,9]. Moreover, serum from individuals with β-thalassemia inhibits hepcidin mRNA expression in hepatoma cells[10]. Hepcidin expression is regulated in hepatocytes by multiple signals, including iron, hypoxia and inflammatory cytokines[7]. Recently, transforming growth factor-β (TGFB) superfamily members have been identified as regulators of hepcidin expression[11–13]. The TGFB superfamily consists of numerous molecules that regulate cellular processes such as growth, differentiation and oncogenesis. Members of the TGFB superfamily include several TGFB proteins, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs) and other proteins[14,15].

We hypothesized that erythroid expansion could influence the regulation of hepcidin expression through systemic release of TGFB superfamily members that are secreted from erythroblasts during human erythropoiesis. A well-characterized *in vitro* model of human erythropoiesis was used as a source of mRNA for developmentally staged, primary human erythroblasts from normal donors[16]. Transcriptional profiles were derived by measuring mRNA expression from less mature erythroblast progenitors (obtained on culture day 7) and more mature, hemoglobinized erythroblast precursors (culture day 14) using cultured cells from 15 healthy donors. Total RNA from these cells was profiled with Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays. For this study, informatic analyses were focused on 54 genes that encode TGFB superfamily members.

Among those 54 genes (**Supplementary Table 1** online), 8 genes from the less mature erythroblasts (**Fig. 1a**) and 11 genes from the mature precursor cells (**Fig. 1b**) were identified by screening for a normalized intensity of ≥ 1. Three genes, *TGFB1* (encoding transforming growth factor-β1), *TWSG1* (encoding twisted gastrulation protein-1) and *GDF15* (encoding growth differentiation factor-15), showed more than a twofold increase in normalized intensity on

[1]Molecular Medicine Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 10 Center Drive, Bethesda, Maryland 20892, USA. [2]Department of Obstetrics and Gynecology, National Naval Medical Center, 8901 Rockville Pike, Bethesda, Maryland 20889, USA. [3]Laboratory Medicine and Pathology, Children's National Medical Center, 111 Michigan Ave. NW, Washington, DC 20010, USA. [4]Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 10 Center Drive, Bethesda, Maryland 20892, USA. [5]Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA. [6]Hematology Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, USA. [7]Departments of Medicine and Pathology, University of California, Los Angeles, 10833 Le Conte Avenue, Los Angeles, California 90095, USA. [8]Department of Transfusion Medicine, National Institutes of Health, 10 Center Drive, Bethesda, Maryland 20892, USA. [9]Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University, Salaya, Phuttamonthon 4 Rd., Phuttamonthon, Nakornpathom 73170, Thailand. Correspondence should be addressed to J.L.M. (jm7f@nih.gov).

Received 28 February; accepted 5 July; published online 26 August 2007; doi:10.1038/nm1629

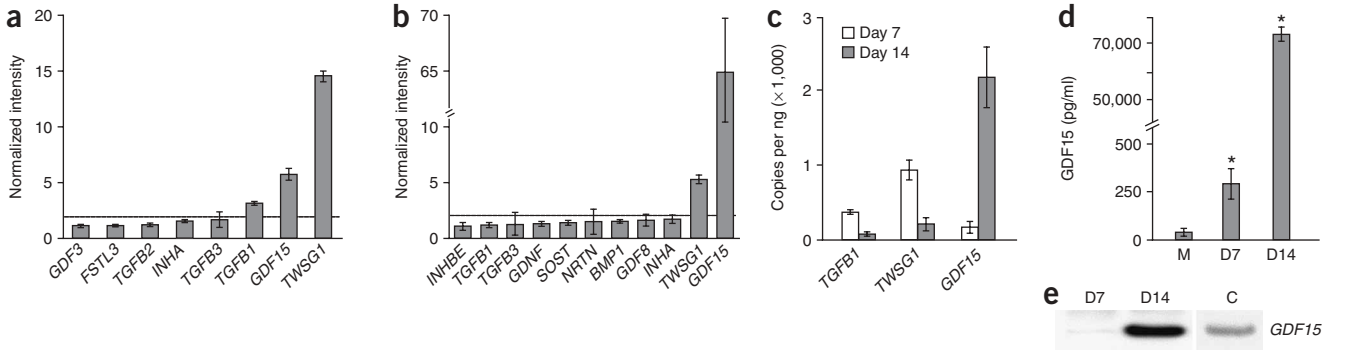

Figure 1 Transcriptional profiling of TGFB superfamily members during erythropoiesis. Genes encoding the TGFB superfamily members with >1-fold changes of normalized intensity (*y*-axis) are shown. (**a**,**b**) Values from cells obtained on culture day 7 (**a**) and day 14 (**b**). The horizontal dashed line in each panel indicates a twofold increase in normalized intensity level. Data pertaining to duplicate probe sets and the remaining TGFB members (<1-fold changes) are shown in **Supplementary Table 1**. The results shown represent the mean values from five pools (three donors per pool) of erythroid progenitors with standard error bars. (**c**) Gene expression of *TGFB1*, *TWSG1* and *GDF15* from hematopoietic progenitor cells cultured for 7 d (open bar) and 14 d (closed bar) were analyzed by quantitative PCR (*y*-axis; copy number per ng of total RNA). Triplicate data are expressed as means ± s.e.m. (**d**) Concentration of GDF15 protein in the medium supernatants sampled on culture days 7 and 14 compared with unconditioned medium (M). (**e**) Immunoblot analysis for GDF15 from cells obtained on culture days 7 and 14 with positive control (C). Results from experiments using three independent donors' cells are represented with standard error bars. Asterisks signify $P < 0.05$.

culture day 7. Two of those genes, *TWSG1* and *GDF15*, also showed a more than twofold change on culture day 14; the increase in *GDF15* expression, which was 64-fold higher as compared to the control, was particularly striking. These results demonstrate that *TGFB1* and *TWSG1* gene expression occurs early during erythroblast maturation, contrasting with the more sustained increase in *GDF15* expression in more mature, hemoglobinized erythroblasts. TGFB1 and GDF15 are ligands for TGF-β receptor signaling. TWSG1 can function as a BMP agonist or antagonist in skeletal and craniofacial development in mice[17]; however, no functional role has been described for this protein in humans.

To examine the expression of these three genes (*TGFB1*, *TWSG1* and *GDF15*) in primary erythroblasts, quantitative PCR, ELISA and immunoblot analyses were performed using primary erythroblasts cultured for 7 and 14 d. On day 7, quantitative PCR analysis showed that the transcript copy numbers (per ng of RNA) were 370 ± 10, 930 ± 140, and 160 ± 78 for *TGFB1*, *TWSG1* and *GDF15*, respectively. Consistent with the array data, the copy numbers of *TGFB1* and *TWSG1* mRNA were both reduced by day 14 in the hemoglobinized cells. However, *GDF15* mRNA expression was significantly increased on day 14 (2,200 ± 410 copies/ng of RNA, $P = 0.025$; **Fig. 1c**). ELISA analyses were performed to determine whether secreted GDF15 was also detectable in the erythroblast culture supernatants. The supernatant concentrations of GDF15 protein were 280 ± 80 pg/ml on day 7 and 74,000 ± 2,580 pg/ml on day 14 (**Fig. 1d**). Immunoblot analyses showed high-level expression of GDF15 protein on day 14 (**Fig. 1e**); TGFB1 and TWSG1 proteins were detected at very low levels on day 7 and not detected on day 14 (data not shown). GDF15 protein was not detected in peripheral blood erythrocytes from healthy volunteers or from individuals with thalassemia (data not shown). Hence, data from the array, PCR, ELISA and immunoblot assays were consistent in demonstrating that GDF15 is expressed and secreted from erythroid progenitor cells as they differentiate and accumulate hemoglobin.

We focused on GDF15 because of the relatively low expression of TGFB1 and TWSG1 as compared to GDF15 observed in cultured erythroblasts (**Fig. 1**), and because of the reported lack of increased TGFB1 in β-thalassemia major patients[18]. The robust expression and secretion of GDF15 from primary erythroblasts *in vitro* suggests that elevated amounts of GDF15 might be detected in the blood of people with β-thalassemia as a result of the marked expansion of erythroblasts in the bone marrow[2]. GDF15 concentrations were measured in venous blood from healthy volunteers, several control patient groups and patients with symptomatic thalassemia (**Fig. 2a**). The mean GDF15 concentration in ten healthy adult volunteers was 450 ± 50 pg/ml. By comparison, GDF15 was significantly elevated ($P = 0.028$) to mean values of 2,300 ± 550 pg/ml in seven cord blood samples from healthy individuals. Blood samples from nine patients with renal cell carcinoma (RCC) were included because GDF15 is known to be expressed in the kidney and to be more highly expressed in some types of cancer[19]; the GDF15 concentrations in these patients (1,100 ± 150 pg/ml) were significantly elevated ($P = 0.040$). GDF15 levels in blood from a population of 21 people being treated for hereditary hemochromatosis were not significantly increased compared to those of healthy controls (720 ± 50 pg/ml; $P = 0.059$), as would be expected because iron overload occurs for reasons not thought to be related to erythropoiesis in hereditary hemochromatosis. Among the patient groups with hemoglobin abnormalities, GDF15 concentrations in patients with sickle cell anemia were elevated (880 ± 160 pg/ml), but not to a statistically significant degree relative to the healthy volunteers ($P = 0.17$). GDF15 concentrations were also measured in serum from ten randomly selected patients with myelodysplastic syndromes (MDS) before bone marrow transplantation. GDF15 concentrations in those MDS patients were elevated but not significantly increased compared to healthy controls (3,400 ± 3,500 pg/ml; $P = 0.089$).

GDF15 concentrations measured in individuals with a mutation in a single globin allele (single copy carriers of α- or β-thalassemia or variant hemoglobin genes; not associated with anemia or iron overload) (Trait, **Fig. 2a**) were also increased, but not to a statistically significant degree (830 ± 100 pg/ml; $P = 0.12$). In contrast, concentrations of GDF15 in 20 patients with α-thalassemia syndrome (α-Thal) were significantly elevated (5,900 ± 1,200; $P = 0.0015$), as were those in 40 patients with β-thalassemia syndrome (β-Thal; $P = 0.00010$). The mean and median increases in GDF15 concentration in the β-thalassemia patients were substantial (mean: 66,000 ± 9,600 pg/ml, median: 48,000 pg/ml).

Correlations between GDF15 concentration and markers of erythropoiesis were determined using blood from patients with α− or

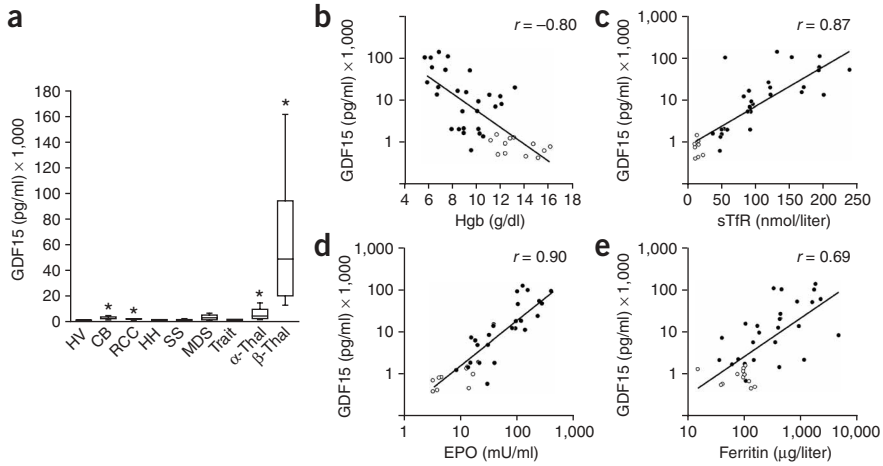

Figure 2 GDF15 concentration in human blood and correlation with concentrations of hemoglobin, erythropoietin, soluble transferrin receptor and ferritin in blood from thalassemia patients. (**a**) GDF15 concentration in blood from healthy volunteers and individuals with various blood abnormalities was analyzed and is depicted using a median value and quartile display. Box plot showing GDF15 concentration in blood from healthy volunteers (HV; $n = 10$), cord blood (CB; $n = 7$), and patients with renal cell carcinoma (RCC; $n = 9$), hereditary hemochromatosis (HH; $n = 21$), sickle cell anemia (SS; $n = 13$), myelodysplastic syndromes (MDS; $n = 10$), α- or β- thalassemia trait (Trait; $n = 12$), α-thalassemia syndromes (α-Thal; $n = 20$) or β-thalassemia syndromes (β-Thal; $n = 40$). $^*P < 0.05$. (**b**) Correlation between GDF15 and hemoglobin levels (Hgb) ($n = 40$, $P < 0.01$). (**c**) Correlation between GDF15 and sTfR levels ($n = 36$, $P < 0.01$). (**d**) Correlation between GDF15 and EPO levels ($n = 36$, $P < 0.01$). (**e**) Correlation between GDF15 and ferritin levels ($n = 39$, $P < 0.01$) in patients with α-thalassemia, β-thalassemia or thalassemia trait (who had received no prior transfusion or chelation therapy; see also **Supplementary Table 2**) as determined by Spearman's correlation. In **b**–**e**, thalassemia syndromes are shown as closed circles and thalassemia traits are shown as open circles. The regression lines are plotted.

β-thalassemia syndromes and thalassemia trait who had undergone no prior transfusion or chelation therapy (**Fig. 2b**–**e**). The clinical profiles of these 40 patients are summarized in **Supplementary Table 2** online. Soluble transferrin receptor (sTfR) was measured as a surrogate for hepcidin, because urine samples were not collected as part of this study, and because sTfR levels have been shown previously to correlate with hepcidin mRNA levels from liver tissues in thalassemia populations[9]. sTfR, erythropoietin and ferritin levels all positively correlated with GDF15 levels, and hemoglobin levels showed a negative correlation.

On the basis of the high concentrations of GDF15 observed in the blood of β-thalassemia patients, as well as of the positive correlation between GDF15 and sTfR and ferritin levels (**Fig. 2c**,**e**), we examined the effect of GDF15 treatment on hepcidin mRNA expression in hepatic cells. For this purpose, quantitative PCR specific for hepcidin mRNA was performed with RNA isolated from primary hepatocytes and cells from a human hepatoma cell line (HuH-7) treated with GDF15 for 24 h (**Fig. 3a**). Addition of recombinant GDF15 to the cells at a concentration normally found in human peripheral blood (100–1,000 pg/ml) increased hepcidin mRNA expression significantly ($P < 0.05$), by about 1.5-fold, as compared to untreated cells. Dosing of GDF15 to even higher concentrations (10,000–100,000 pg/ml), similar to those in people with β-thalassemia, suppressed hepcidin mRNA expression as compared to the physiological range of GDF15 ($P < 0.001$). The pattern of GDF15-mediated regulation of hepcidin expression was similar between the hepatoma cell line and the primary human hepatocytes, with no overt cellular toxicities observed. Preliminary experiments showed no effects on iron accumulation or hepcidin expression in mice transgenic for human GDF15 and an inability of human GDF15 to suppress hepcidin expression in mouse hepatocytes (T.E.E. and T.G., unpublished results).

To determine whether GDF15 in the blood of β-thalassemia patients can regulate hepcidin expression, we measured hepcidin mRNA expression in primary hepatocytes exposed to the sera of eight individuals with β-thalassemia (**Fig. 3b**). The mean final concentration of GDF15 in the culture medium containing β-thalassemia serum was 9,000 ± 1,300 pg/ml. Hepcidin mRNA expression in the hepatocytes was significantly reduced ($P = 0.0005$) in medium

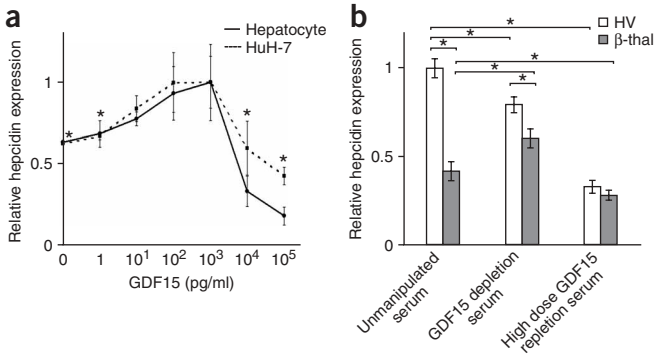

Figure 3 Regulation of hepcidin mRNA expression by GDF15. (**a**) Dose response of hepcidin mRNA levels to GDF15 was determined in human primary hepatocytes (solid line) and the HuH-7 cell line (broken line) grown in medium containing GDF15 at final concentrations of 0, 1, 10, 100, 1,000, 10,000, and 100,000 pg/ml. Relative hepcidin expression (normalized with GAPDH mRNA) is shown on the *y*-axis. mRNA expression at a physiological level of GDF15 was assigned a value of 1 for comparison. In each case, quantitative real-time PCR was performed in triplicate (means and standard error bars are shown). Statistical significance was determined relative to 1,000 pg/ml values; $^*P < 0.05$. (**b**) Hepcidin mRNA expression in primary hepatocytes treated with culture medium containing 10% of either serum from healthy volunteers ($n = 4$; assay performed in triplicate; open bars) or serum from patients with β-thalassemia ($n = 8$; assay performed in triplicate; closed bars). Relative hepcidin expression (normalized by GAPDH mRNA) is shown on the *y*-axis. Results obtained with serum from the healthy volunteers were assigned a value of 1 for comparison. For unmanipulated serum, the final mean concentrations of GDF15 in the culture medium were 60 and 9,000 pg/ml for control and β-thalassemia serum, respectively; for GDF15-depleted serum, the final mean concentrations of GDF15 in the culture medium were 1 and 90 pg/ml for control and β-thalassemia serum, respectively; and for high-dose GDF15 repletion serum, the final mean concentrations of GDF15 in the culture medium were 100,000 and 100,000 pg/ml for control and β-thalassemia serum, respectively. The mean values with standard error bars are shown. $^*P < 0.05$.

containing thalassemia serum when compared to medium containing serum from healthy volunteer controls (mean GDF15 concentration 620 ± 130 pg/ml). GDF15 was depleted by immunoprecipitation and the depletion confirmed by ELISA (after depletion, the final concentrations of GDF15 in the media containing healthy volunteer control and thalassemia sera were 1.2 ± 0.3 pg/ml and 92.3 ± 15.2 pg/ml, respectively). After GDF15 depletion, the amount of hepcidin mRNA in cells grown in medium containing control serum was decreased, whereas the amount of hepcidin mRNA in cells grown in medium containing β-thalassemia serum was significantly increased ($P = 0.031$). However, the level of hepcidin mRNA in cultures containing GDF15-depleted β-thalassemia serum did not reach the level detected in cultures containing GDF-depleted control serum. Reconstitution of both types of depleted sera with a high concentration of GDF15 (100,000 pg/ml) resulted in significant ($P < 0.0001$) suppression of hepcidin mRNA expression. These results are consistent with the dose response seen with GDF15 treatment of hepatocytes (**Fig. 3a**) and suggest that the GDF15 in β-thalassemia serum is biologically active.

These findings indicate that GDF15 is secreted at high levels by erythroblasts, and we propose that GDF15 may normally function in the erythroid niche. Although mildly elevated expression of GDF15 has been reported in a variety of conditions related to tissue injury and stress, the high level of expression noted here in patients with thalassemia examined is unusual. Such high levels are also measured during pregnancy, when there is a progressive rise in maternal GDF15 concentration from a normal level to >30,000 pg/ml in the third trimester[20]. The high amount of GDF15 expressed during pregnancy has been attributed to placental expression[21], and this may explain the elevation in GDF15 levels seen in plasma from cord blood (**Fig. 2**; 2,300 ± 550 pg/ml. There is increased iron absorption during pregnancy, and consistent with the results reported here, pregnancy is also associated with suppression of hepcidin expression in animal models[22]. Elevated serum GDF15 concentrations have been reported in a variety of cancers[23], but are rarely higher than 5,000 pg/ml[24], similar to the results shown here in individuals with renal cell carcinoma.

GDF15 is synthesized as a 40-kDa propeptide that is cleaved to release a 25-kDa mature protein into the circulation[19]. GDF15 was originally thought to function as a macrophage inhibitor, but further analyses suggested other possible roles for GDF15. Because *GDF15* is targeted by the tumor suppressor p53 (ref. 25), its expression in humans is increased by stress conditions such as anoxia, cancer and tissue injury. *GDF15* expression is also increased by anoxia through an undefined mechanism that is p53-independent. Studies in cardiomyocytes suggest that GDF15 activates Smad2/3 signaling[26]. GDF15 is generally thought to cause apoptosis and behave as a tumor suppressor protein in model systems. *Gdf15*-transgenic mice have shown a reduced body weight, bony abnormalities and resistance to neoplasia[19]. In *Gdf15*$^{-/-}$ mice, no phenotype has been observed[27]. Although our preliminary evidence suggests that human GDF15 does not suppress hepcidin mRNA expression in mouse hepatocytes, additional mouse studies will be required to determine the level of similarity between mice and humans with regard to the role of GDF15 in regulating hepcidin expression.

We propose that in humans with symptomatic thalassemia syndromes, iron regulation is mediated by erythroblast secretion of GDF15 (**Fig. 4**). Anemia resulting from ineffective erythropoiesis and hemolysis results in lifelong tissue hypoxia that drives the extreme overexpansion of erythropoiesis in individuals with these syndromes; GDF15 and possibly other proteins are increased in the circulation in these individuals. Iron itself does not cause the increased expression of

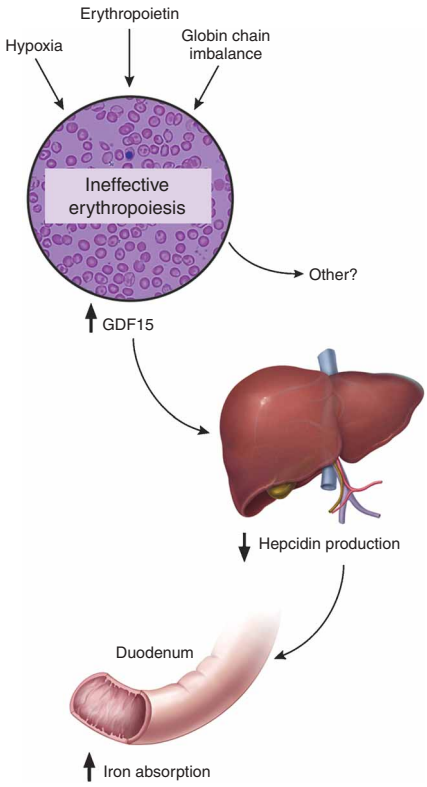

Figure 4 Model for iron regulation in thalassemia patients.

GDF15, as shown by the lack of increased GDF15 levels seen in the blood of patients with primary hemochromatosis. GDF15 produced by erythroblasts circulates to the liver and suppresses hepcidin expression; as a result, increased amounts of dietary iron are absorbed, and secondary hemochromatosis develops over the lifetime of the patient. The reduction in hepcidin expression in hepatocytes induced by high levels of GDF15 (**Fig. 3**) is consistent with the reduction in urinary hepcidin detected *in vivo* in patients with thalassemia major[8,9,28]. However, in our study, GDF15 did not suppress hepcidin expression at low concentrations or cause complete suppression of hepcidin even at very high concentrations. These findings suggest that GDF15 is not the sole regulator of hepcidin expression, but instead contributes to the suppression of hepcidin in the pathological setting of thalassemia.

Marrow expansion, tissue hypoxia, and erythroblast apoptosis caused by globin chain imbalances may all be needed to induce the extremely high concentrations of GDF15 detected in the serum of β-thalassemia patients. In contrast, the main pathogenic features of sickle cell anemia are hemolysis and occlusive sickling, rather than ineffective erythropoiesis, and patients with this condition have no significant increases in GDF15. Notably, iron deficiency, rather than iron overload, is very common among nontransfused patients with sickle cell anemia[29]. The dramatic difference in the GDF15 levels and iron stores between these two hemoglobinopathy patient populations suggests that ineffective erythropoiesis, such as occurs in thalassemias and other iron-loading anemias, may be a key mechanism for the greatly increased expression of GDF15. In the small number of MDS patients described here, GDF15 concentrations were elevated, but were much lower than those detected in patients with thalassemia syndromes. Further studies of GDF15 expression levels among other patient groups with ineffective erythropoiesis or unexplained iron

loading pathology—including patients with specific MDS subtypes, sideroblastic anemia, congenital dyserythropoietic anemia, pernicious anemia and a variety of liver diseases—are underway to determine whether GDF15 is overexpressed to the high levels detected in thalassemia. In the β-thalassemia syndromes, the combination of extensive bone-marrow expansion and severely ineffective erythropoiesis may be necessary to produce the unusually high expression of GDF15. Because the iron-loading pathology of thalassemia has been associated with bone pathology, it is possible that GDF15, as a member of the bone morphogenic family of proteins, also contributes to the severe bone abnormalities of thalassemia. As such, we propose that GDF15 represents a biomarker as well as a potential therapeutic target for β-thalassemia syndromes.

METHODS

Human studies. Studies were performed after informed consent was obtained according to approval from the National Institute of Diabetes, Digestive and Kidney Diseases Institutional review board or under an exemption from Title 45, Part 46 of the US Department of Health and Human Services Code of Federal Regulations.

Healthy donors were recruited for the $CD34^+$ mobilizations used for the array studies. For other experiments, venous blood was gathered from the authors' frozen stocks. Study samples were collected from healthy volunteers ($n = 10$), cord blood from uncomplicated term deliveries ($n = 7$), and individuals with renal cell carcinoma ($n = 9$), hereditary hemochromatosis ($n = 21$), sickle cell anemia ($n = 13$), myelodysplastic syndromes ($n = 10$), thalassemia trait ($n = 12$), α-thalassemia syndromes ($n = 20$) and β-thalassemia syndromes ($n = 40$).

Transcriptional profiling. We cultured $CD34^+$ cells from 15 separate healthy donors in medium containing 4 U/ml erythropoietin (EPO) (Amgen) as described previously[16]. On culture days 7 and 14, the cells were sampled, counted and examined microscopically and by flow cytometry to confirm erythroblast differentiation. Total RNA was extracted from the cells and assayed with Bioanalyzer (Agilent Technologies) and then pooled (five pools; three donors each). Microarray analyses were performed using 2 μg total RNA from each pool with one cycle of complementary RNA amplification, using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) according to the manufacturer's protocol. After we performed the hybridization and washing steps, we scanned the microarray chips with MAS 5.0 software. Control studies were done to confirm array-based gene expression patterns by quantitative PCR (data not shown). Only Affymetrix grade A probe sets were selected from the manufacturer's database (NetAffx web database; http://www.affymetrix.com/analysis/index.affx). Grade A probe sets for the GDF6, GDF7 and A2M genes were not identified. As a result, a subset of 74 probe sets assigned to 54 members of the TGFB superfamily genes was studied. Signal normalization and probe detection levels (normalized intensities) were determined according to the manufacturer's default settings using GeneSpring GX 7.3 software (Agilent Technologies). The normalized intensities of these probe sets are shown in **Supplementary Table 1** online.

Quantitative PCR analysis for gene expression. Total RNA was isolated using TRIzol LS Reagent (Invitrogen) and then treated with 1 U DNase I (Ambion) at 37 °C for 15 min. The first-strand cDNA was generated using Superscript III (Invitrogen) with oligo-dT_{20} primer from the same amount of total RNA following the manufacturer's protocol. A quantitative real-time PCR assay was carried out with gene-specific TaqMan probes for human *GDF15*, *TWSG1*, and *TGFB1* labeled with 6-carboxyfluorescein (FAM) dye using a 7700 Sequence Detector (Applied Biosystems). All probes are designed to span exon junctions in the fully processed message in order to prevent the detection of contaminating genomic DNA. Each PCR reaction was optimized to ensure that a single PCR product was amplified and that no product corresponding to primer-dimer pairs was present. Blank controls that did not contain cDNA were run in parallel. PCR amplification was performed in triplicate using a 96-well plate format. The PCR cycling conditions were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 PCR cycles (95 °C for 15 s, 60 °C for 1 min). A plasmid encoding each gene template was used to generate the standard curve (20–20,000,000 copies) for determination of copy number.

Immunoblot and ELISA analysis. Cells cultured for 7 and 14 d were obtained and protein was extracted using mammalian protein extraction reagent (Pierce Biotechnology) as recommended by the manufacturer. The protein extracts were electrophoresed (25 μg/lane) through a sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were probed with antibody to human GDF15, antibody to human TGFB1 antibody, and antibody to mouse Tsg (the mouse homolog to TWSG1) antibody (R&D Systems); bound antibodies were detected with secondary horseradish peroxidase (HRP)-conjugated antibodies (Amersham Pharmacia, Piscataway, NJ). Mature human TWSG1 shares 97.5% amino acid sequence similarity with mouse Tsg and the polyclonal antibody for mouse Tsg showed some cross-reactivity with human TWSG1. Human recombinant proteins for GDF15 and TGFB1 (R&D Systems) and TWSG1 (Abnova) were used as positive controls for each assay. The GDF15 ELISA used for quantification of GDF15 in cell culture media and blood samples was performed with DuoSet ELISA for human GDF15 (R&D Systems) following the manufacturer's protocol. The ELISA assay for sTfR in blood samples was performed with Human sTfR Quantikine IVD ELISA Kit (R&D Systems) following the manufacturer's protocol.

Hepcidin assays. Hepcidin assays with recombinant proteins were performed under the conditions described[30]. Human primary hepatocytes (Liver Tissue Procurement and Distribution System) were cultured in human hepatocyte maintenance medium (Lonza). The human hepatoma cell line (HuH-7) was grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. All cultures were maintained at 37 °C under 5% CO_2. Human GDF15 (R&D Systems) was used to determine the effects on hepcidin mRNA expression in the cells. Cells were grown in culture plates (BD Bioscience) and incubated with the recombinant proteins for 24 h.

For assays using human serum, the effects of GDF15 depletion on hepcidin expression were assayed based upon previously reported methods[10]. For GDF15-depleted serum, we removed GDF15 from human serum using a Protein G immunoprecipitation kit (Sigma-Aldrich) with human GDF15-specific monoclonal antibody (R&D Systems). The concentration of GDF15 in the serum after the depletion was confirmed with DuoSet ELISA for human GDF15 (R&D Systems). Recombinant human GDF15 protein (R&D Systems) was added to the depleted serum for reconstitution. In each case, final concentrations of 10% human serum were present in the culture medium.

Statistical analysis. Replicate data are expressed as means ± s.e.m. with significance calculated by Student's *t*-test. GDF15 concentrations in blood samples were expressed as median and quartile values. Comparisons of the concentrations of GDF15 in blood samples between the groups of healthy volunteers and patients were performed with the nonparametric Kruskal-Wallis test and then the Mann-Whitney test with Bonferroni correction. Correlations between the various parameters were calculated using the Spearman's correlation.

The microarray data were deposited in the public domain (National Center for Biotechnology Information Gene Expression Omnibus Gene Expression Omnibus accession number, GSM190964–GSM190973).

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

The National Institutes of Health Department of Transfusion Medicine, National Institute of Diabetes and Digestive and Kidney Diseases Microarray Core Laboratory, and S. Strom, J. Goodnough, G. Moser and M. Ehinger performed or assisted with studies involving hepatocytes and transgenic mice. We thank C. Deng, C. Philpott, and A. Schechter for critical reading of the manuscript. This research was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases.

AUTHOR CONTRIBUTIONS

T. T., manuscript writing, performed experiments, assembly of data. N.V.B., collection and assembly of array data. P.A.O., collection of clinical samples and

data. S.-H.G., provision of study material, array data analysis. P.S., collection of clinical samples and data. Y.T.L., collection and assembly of array data. J.W.M., collection of clinical samples. C.H.R., collection of clinical samples and data. N.L.C.L., collection of clinical samples and data. R.-H.W., expertise with hepatic iron assessment. T.E., expertise with GDF15 transgenic mouse. R.C., collection of clinical samples and data. T.G., mouse GDF15 analyses, manuscript revision. S.F.L., collection of clinical samples and data. S.F., collection of clinical samples and data. J.L.M., manuscript writing, experimental conception and design, assisted and supervised research team.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

Published online at http://www.nature.com/naturemedicine
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Rund, D. & Rachmilewitz, E. β-thalassemia. *N. Engl. J. Med.* **353**, 1135–1146 (2005).
2. Pootrakul, P. *et al.* A correlation of erythrokinetics, ineffective erythropoiesis, and erythroid precursor apoptosis in Thai patients with thalassemia. *Blood* **96**, 2606–2612 (2000).
3. Nathan, D.G. Thalassemia as a proliferative disorder. *Medicine (Baltimore)* **43**, 779–782 (1964).
4. Weatherall, D.J. & Clegg, J.B. *The Thalassaemia Syndromes (4th ed.)* (Blackwell Scientific, Oxford, 2001).
5. Nemeth, E. & Ganz, T. Regulation of iron metabolism by hepcidin. *Annu. Rev. Nutr.* **26**, 323–342 (2006).
6. Donovan, A., Roy, C.N. & Andrews, N.C. The ins and outs of iron homeostasis. *Physiology (Bethesda)* **21**, 115–123 (2006).
7. Ganz, T. Hepcidin—a regulator of intestinal iron absorption and iron recycling by macrophages. *Best Pract. Res. Clin. Haematol.* **18**, 171–182 (2005).
8. Papanikolaou, G. *et al.* Hepcidin in iron overload disorders. *Blood* **105**, 4103–4105 (2005).
9. Kattamis, A. *et al.* The effects of erythropoetic activity and iron burden on hepcidin expression in patients with thalassemia major. *Haematologica* **91**, 809–812 (2006).
10. Weizer-Stern, O. *et al.* Downregulation of hepcidin and haemojuvelin expression in the hepatocyte cell–line HepG2 induced by thalassaemic sera. *Br. J. Haematol.* **135**, 129–138 (2006).
11. Wang, R.H. *et al.* A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. *Cell Metab.* **2**, 399–409 (2005).
12. Babitt, J.L. *et al.* Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat. Genet.* **38**, 531–539 (2006).
13. Truksa, J., Peng, H., Lee, P. & Beutler, E. Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6. *Proc. Natl. Acad. Sci. USA* **103**, 10289–10293 (2006).
14. Massague, J. TGF-β signal transduction. *Annu. Rev. Biochem.* **67**, 753–791 (1998).
15. de Caestecker, M. The transforming growth factor-β superfamily of receptors. *Cytokine Growth Factor Rev.* **15**, 1–11 (2004).
16. Wojda, U., Noel, P. & Miller, J.L. Fetal and adult hemoglobin production during adult erythropoiesis: coordinate expression correlates with cell proliferation. *Blood* **99**, 3005–3013 (2002).
17. Yamamoto, Y. & Oelgeschlager, M. Regulation of bone morphogenetic proteins in early embryonic development. *Naturwissenschaften* **91**, 519–534 (2004).
18. Sritippayawan, S. *et al.* Restrictive lung disease and serum TGF-β1 in thalassemia major children. *Asian Pac. J. Allergy Immunol.* **23**, 121–126 (2005).
19. Eling, T.E. *et al.* NSAID activated gene (NAG-1), a modulator of tumorigenesis. *J. Biochem. Mol. Biol.* **39**, 649–655 (2006).
20. Marjono, A.B. *et al.* Macrophage inhibitory cytokine-1 in gestational tissues and maternal serum in normal and pre-eclamptic pregnancy. *Placenta* **24**, 100–106 (2003).
21. Hromas, R. *et al.* PLAB, a novel placental bone morphogenetic protein. *Biochim. Biophys. Acta* **1354**, 40–44 (1997).
22. Millard, K.N., Frazer, D.M., Wilkins, S.J. & Anderson, G.J. Changes in the expression of intestinal iron transport and hepatic regulatory molecules explain the enhanced iron absorption associated with pregnancy in the rat. *Gut* **53**, 655–660 (2004).
23. Welsh, J.B. *et al.* Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. *Proc. Natl. Acad. Sci. USA* **100**, 3410–3415 (2003).
24. Koopmann, J. *et al.* Serum markers in patients with resectable pancreatic adenocarcinoma: macrophage inhibitory cytokine 1 versus CA19–9. *Clin. Cancer Res.* **12**, 442–446 (2006).
25. Yang, H., Filipovic, Z., Brown, D., Breit, S.N. & Vassilev, L.T. Macrophage inhibitory cytokine-1: a novel biomarker for p53 pathway activation. *Mol. Cancer Ther.* **2**, 1023–1029 (2003).
26. Xu, J. *et al.* GDF15/MIC-1 functions as a protective and antihypertrophic factor released from the myocardium in association with SMAD protein activation. *Circ. Res.* **98**, 342–350 (2006).
27. Hsiao, E.C. *et al.* Characterization of growth-differentiation factor 15, a transforming growth factor β superfamily member induced following liver injury. *Mol. Cell. Biol.* **20**, 3742–3751 (2000).
28. Kearney, S.L. *et al.* Urinary hepcidin in congenital chronic anemias. *Pediatr. Blood Cancer* **48**, 57–63 (2007).
29. Rao, K.R., Patel, A.R., McGinnis, P. & Patel, M.K. Iron stores in adults with sickle cell anemia. *J. Lab. Clin. Med.* **103**, 792–797 (1984).
30. Inamura, J. *et al.* Upregulation of hepcidin by interleukin-1β in human hepatoma cell lines. *Hepatol. Res.* **33**, 198–205 (2005).