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The Small Molecule, Genistein, Increases Hepcidin Expression in Human Hepatocytes

Aileen W. Zhen,¹ Nancy H. Nguyen,¹ Yann Gibert,² Shmulik Motola,³ Peter Buckett,⁴ Marianne Wessling-Resnick,⁵ Ernest Fraenkel,⁶ and Paula G. Fraenkel¹

Hepcidin, a peptide hormone that decreases intestinal iron absorption and macrophage iron release, is a potential drug target for patients with iron overload syndromes because its levels are inappropriately low in these individuals. Endogenous stimulants of Hepcidin transcription include bone morphogenic protein 6 (BMP6) and interleukin-6 (IL-6) by effects on mothers against decapentaplegic homolog (Smad)4 or signal transducer and activator of transcription (Stat)3, respectively. We conducted a small-scale chemical screen in zebrafish embryos to identify small molecules that modulate hepcidin expression. We found that treatment with the isoflavone, genistein, from 28-52 hours postfertilization in zebrafish embryos enhanced Hepcidin transcript levels, as assessed by whole-mount in situ hybridization and quantitative real-time reverse-transcriptase polymerase chain reaction. Genistein's stimulatory effect was conserved in human hepatocytes: Genistein treatment of HepG2 cells increased both Hepcidin transcript levels and promoter activity. We found that genistein's effect on Hepcidin expression did not depend on estrogen receptor signaling or increased cellular iron uptake, but was impaired by mutation of either BMP response elements or the Stat3-binding site in the Hepcidin promoter. RNA sequencing of transcripts from genistein-treated hepatocytes indicated that genistein up-regulated 68% of the transcripts that were up-regulated by BMP6; however, genistein raised levels of several transcripts involved in Stat3 signaling that were not up-regulated by BMP6. Chromatin immunoprecipitation and ELISA experiments revealed that genistein enhanced Stat3 binding to the Hepcidin promoter and increased phosphorylation of Stat3 in HepG2 cells. Conclusion: Genistein is the first small-molecule experimental drug that stimulates Hepcidin expression in vivo and in vitro. These experiments demonstrate the feasibility of identifying and characterizing small molecules that increase Hepcidin expression. Genistein and other candidate molecules may subsequently be developed into new therapies for iron overload syndromes. (HEPATOLOGY 2013;58:1315-1325)

Hepcidin is a transcriptionally regulated peptide hormone¹ that is expressed primarily in the liver and excreted in urine. It is up-regulated in response to inflammation²,³ or iron overload⁴ and down-regulated in response to increased erythropoiesis, iron deficiency, or hypoxia.² Hepcidin decreases intestinal iron absorption and macrophage iron release by causing internalization of the iron exporter, ferroportin1.⁵⁻⁷ Patients with hereditary hemochromatosis⁸ or thalassemia⁹⁻¹¹ exhibit inappropriately low levels of hepcidin and increased intestinal iron absorption, despite the presence of systemic iron overload¹². Although treatment for iron overload is currently based on removal of blood or administration of iron chelators, it may be possible to

Abbreviations: Abs, antibodies; BMP, bone morphogenic protein; BRE, BMP response element; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FKPM, fragments per kilobase of exon per million fragments mapped; GO, Gene Ontology; hpf, hours postfertilization; Ig, immunoglobulin; IL-6, interleukin-6; IRF9, interferon regulatory factor 9 gene; kb, kilobase; KEGG, Kyoto Encyclopedia of Genes and Genomes; MEM, minimal essential media; mRNA, messenger RNA; pRL-CMV, Renilla luciferase under control of the cytomegalovirus promoter; pStat3, phospho-Stat3; RNA-seq, RNA sequencing; RT-PCR, reverse-transcriptase polymerase chain reaction; Smad, mothers against decapentaplegic homolog; SOCS3, suppressor of cytokine signaling 3 gene; Stat3, signal transducer and activator of transcription; TGF-β, transforming growth factor beta; VEGFα, vascular endothelial growth factor A gene; WT, wild type.

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prevent iron overload in patients with genetic predisposition if nontoxic small molecules can be administered that increase transcription of Hepcidin.

Iron overload and inflammation stimulate hepcidin expression by triggering the mothers against decapentaplegic homolog (Smad)-signaling or signal transducer and activator of transcription (Stat)-signaling pathways, respectively. It has been demonstrated that exposing human hepatocytes to bone morphogenic proteins (BMPs) up-regulates Hepcidin transcription by increasing Smad4 binding at Smad4-binding motifs, termed BMP response elements (BREs), in the Hepcidin promoter.13-16 BMPs are members of the transforming growth factor beta (TGF-β) family that signal by binding to transmembrane receptor complexes with serine-threonine kinase activity. Recent studies in mouse models indicate that BMP6 is the most likely physiologic regulator of hepcidin transcription in response to iron loading. Inflammatory stimuli, on the other hand, trigger increased serum interleukin-6 (IL-6) levels.22 IL-6 stimulates Hepcidin expression through increased Stat3 binding to a Stat3-responsive element in the Hepcidin promoter.24-27

We have developed the zebrafish embryo (Danio rerio) as an in vivo model to study hepcidin expression. Hepcidin expression begins at 36 hours postfertilization (hpf) in the zebrafish embryo and is responsive to iron levels and BMPs during embryonic development.28 To demonstrate that zebrafish embryos can be used to identify small-molecule modulators of Hepcidin expression, we screened a small number of naturally occurring isoflavones and related molecules for their effect on Hepcidin expression. We chose to evaluate isoflavones because they are nontoxic and are known to have kinase inhibitory actions.29 In this way, we identified genistein as the first small-molecule experimental drug to increase Hepcidin expression in vivo. We found that genistein also increased Hepcidin expression in cultured human hepatocytes (HepG2 cells). Using luciferase reporter assays, RNA sequencing (RNA-seq), and chromatin immunoprecipitation (ChIP), we demonstrated that genistein increases Hepcidin expression in a Smad4-dependent and Stat3-dependent manner.

Materials and Methods

Zebrafish Embryo Chemical Treatment, In Situ Hybridization, and Complementary DNA Preparation. Ethical approval was obtained from the institutional animal care and use committee of Beth Israel Deaconess Medical Center (Animal Welfare Assurance #A3153-01; Boston, MA) in accord with national and international guidelines. Zebrafish were maintained as previously described.30 Pools of 20 embryos were treated either with 7 μM of genistein, genistein, apigenin, daidzein, or estradiol and/or 40 μM of dorsomorphin (all obtained from Sigma-Aldrich, St. Louis, MO) or vehicle alone (1% dimethyl sulfoxide; DMSO) from 28 to 52 hpf. Embryos were then fixed in 4% paraformaldehyde in phosphate-buffered saline for whole-mount in situ hybridization using anti-sense zebrafish hepcidin or FOXA3 probes, as previously described.51 O-dianisidine staining for hemoglobin and flow cytometry were performed as described in the Supporting Materials. Representative embryos were photographed at 100× magnification with a BX51 compound microscope (Olympus, Center Valley, PA) and a Q-capture 5 digital camera (QImaging, Surrey, British Columbia, Canada). Expression analysis was conducted at the specified time points, by anesthetizing pools of 20 embryos with tricaine, followed by storage in RNAlater (Ambion, Life Technologies, Grand Island, NY). RNA extraction and generation of complementary DNA (cDNA) were performed as previously described.6,32

Cell Culture and Chemical Treatment. The human hepatocarcinoma cell line, HepG2 (American Type Culture Collection, Manassas, VA), was maintained in alpha-minimum essential medium (α-MEM)/
Table 1. KEGG Pathway Analysis of Genes That Were Up-Regulated by Genistein

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<th>Term</th>
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<td>37</td>
<td>0.00E-01</td>
<td>RPL18, RPL13, RPS3AP47, RPL35, RPL2, RPL36, RPL37, RPS19P3, RPL38, RPLP0P2, RPS27, RPL32, RPS11P5, RPL1, RPL3, RPL29P26, RPL12, RPL7A, RPL10A, RPS20, RPS21, RPS13P2, RPS23, RPS15P5, RPSA, RPS16P10, RPS9, RPL27, RPL23A, RPSAP8, RSP3P3, RPS6, RPS5, RPL28, RPS8, RPL18A, RPL37A, UBA52</td>
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<tr>
<td>hsa04110/cell cycle</td>
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<td>MAD1L1, FZR1, E2F4, PKMYT1, ANAPC11, SFN, PTG1, ZBTB17, TGFBI, RBX1, MCM7, CDKN2B, TFOP2, MYC, CCNA2, BUB3, CUL1, ANAPC2, CREBBP, CDC20, CDK7, CDC25C, MCM5, CDC25B, CCNB1, CCNB1A, CCNB1D, CCNB1B, VWFAH, CCNB2, PLK1, ANAPC7, AB1, MAD1L2, GADD45B, GADD45A</td>
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<td>hsa05221/acute myeloid leukemia</td>
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<td>HRAS, PPARD, MAP2K1, MAP2K2, RELA, STAT5B, PIK3CD, PIM1, RPS6K2B, BAD, TCF7L2, STAT3, JUP, EIF4EBP1, CCND1, ARAF, IKBKG, RARA, RUNXI, MYC, AKT2</td>
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10% certified endotoxin-free fetal bovine serum (FBS)/1% penicillin-streptomycin (Life Technologies) at 37°C in 5% CO₂. HepG2 cells were seeded onto 12-well tissue-culture-treated plates at a density of 4 × 10⁵ cells per well. Twenty-four hours later, the culture medium was changed to low-serum medium (α-MEM/1% FBS). After 8 hours of equilibration, cells were then treated for the specified time periods with genistein, genistin, apigenin, daizdein, dorsomorphin (each at 10 μM). After 18 or 24 hours of treatment, cells were harvested for extraction of total RNA using the RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. DNA was eliminated by on-column RNase-free DNase treatment. Reverse transcription was performed using RNA (300 ng), oligo-dT, and Superscript II reverse transcriptase (RT; Life Technologies) in a 20-μL reaction, which was later diluted to 100 μL.

Ten percent of each RT reaction was used for a single 50-μL quantitative real-time RT-PCR (polymerase chain reaction) assay using Taqman Universal PCR Master Mix or SYBR Green PCR Master Mix (both from Life Technologies). Primer and probe sequences are given in Supporting Table 1. Transcript levels were calculated as fold increases over the control sample. The described reactions failed to amplify the nontemplate controls or RNA samples that were not treated with RT.

**Transferrin and Nontransferrin-Bound ⁵⁵Fe Uptake Assays and Ferritin Enzyme-Linked Immunosorbent Assay.** Methods for transferrin and nontransferrin-bound ⁵⁵Fe uptake assays and ferritin enzyme-linked immunosorbent assay (ELISA) are provided in the Supporting Materials.

**Dual Luciferase Assay.** HepG2 cells were transfected with pGL4.17 Hepc, 3 kilobases (kb) of the human Hepcidin promoter upstream of the firefly luciferase reporter gene, or mutated versions of the construct with point mutations in the Stat3-binding site, the BREs, or both the BRE-binding and the Stat3-binding site. Additional details are provided in the Supporting Materials. The Hepcidin reporter constructs were donated by Drs. Ganz and Nemeth (Division of Pulmonary and Critical Care, University of California, Los Angeles). As a control, HepG2 cells were cotransfected with Renilla luciferase under control of the cytomegalovirus promoter (pRL-CMV; Promega, Madison, WI). Transiently transfected cells were treated for 6 hours with genistein (10 μM), BMP6 (50 ng/mL), or vehicle (1% DMSO) only. The Dual-Glo Luciferase Assay (Promega) was then performed according to the manufacturer’s instructions.

**Statistical Analysis for Quantitative PCR, Iron Uptake, Luciferase, ChIP, and ELISA.** All data shown are means ± standard error from the mean. Data analysis was performed using Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). For comparisons of
two groups, two-tailed Student t tests were performed comparing experimental treatment to DMSO treatment. For comparisons of three or more groups, Kruskal-Wallis’ test was performed on raw data or natural logs of raw data. If \( P < 0.05 \), pairwise comparisons were made with two-tailed Student t tests. Comparisons where \( P < 0.05 \) were deemed significant.

**RNA-seq.** As described above, HepG2 cells were cultured in 12-well plates, shifted to low-serum conditions, and treated with DMSO (1%), BMP6 (50 ng/mL), or genistein (10 \( \mu \)M) for 18 hours. The numbers of biological replicates were 3, 2, and 3, respectively. Details are provided in the Supporting Materials. Differential expression for BMP6-treated or genistein-treated biological replicates, compared to DMSO-treated controls, was determined using the Cufflinks tool, Cuffdiff. The criteria for differential expression were as follows: a log2 fold-change of >0.58 or \(<-0.58\) and a Q value <0.05, when compared to the control condition. We also required that the differentially expressed genes used for downstream analysis had a fragments per kilobase of exon per million fragments mapped (FPKM) >0.1 in at least one of the conditions. Enrichments in particular biological processes, pathways, and functions were calculated statistically using the DAVID annotation database.34 Prism 5.0 was used to perform linear regression analysis of the correlation between the natural log of gene expression changes measured by RNA-seq and quantitative real-time RT-PCR. All RNA-seq data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo).

**ChIP Followed by Quantitative PCR.** HepG2 cells were seeded on 150-mm\(^2\) plates and cultured to reach a cell density of 5 \( \times \) 10\(^7\) cells per plate. Eight hours before treatment, cells were washed and shifted to low-serum media (\( \alpha \)-MEM/1% FBS). Chemicals were added to achieve either 1% DMSO, 10 \( \mu \)M of genistein, 50 ng/mL of BMP6, or 20 ng/mL of IL-6. After 24 hours of treatment, cells were cross-linked by adding formaldehyde to reach a final concentration of 1%. After reversal of cross-linking, cells were lysed, sonicated, and chromatin was immunoprecipitated with antibody (Ab)-coated Dynabeads Protein G (Life Technologies). Additional details are provided in Supporting Materials. The Abs used were anti-Stat3 (sc-7154) and anti-rabbit immunoglobulin (Ig)G (sc-2027) (both from Santa Cruz Biotechnology, Santa Cruz, CA). DNA was eluted from the beads and used as a template in a quantitative PCR reaction using primers (Supporting Table 1) to amplify the proximal BMP/Stat response element, located 84 nucleotides before the Hepcidin start site.25 The data were reported as fold increase in binding of the specific Ab over binding to nonspecific Ig.

**Stat3 ELISA.** Adherent HepG2 cells (2 \( \times \) 10\(^4\) cells per well of a 96-well plate) were incubated in serum-free \( \alpha \)-MEM for 16 hours, followed by the addition of the following treatments: DMSO (1%); genistein (10 \( \mu \)M); or IL-6 (100 ng/mL). Cells were fixed in 4% formaldehyde after 0-60 minutes of treatment. Detection of Stat3 and phospho-Stat3 (pStat3) was performed using the Stat3 Colorimetric In-Cell ELISA Kit (Thermo Fisher Scientific Pierce, Rockford, IL), according to the manufacturer’s instructions.

**Results**

**Genistein Increases hepcidin Expression in Zebrafish Embryos.** Because we had previously demonstrated evolutionarily conserved aspects of hepcidin regulation in zebrafish embryos,28 we used zebrafish embryos to evaluate naturally occurring isoflavones (genistein and daizdein), genistin (the inactive glucoside form of genistein), or a flavone (apigenin) as potential modulators of mammalian Hepcidin expression. Because genistein, daizdein, and apigenin have estrogenic effects, we also evaluated estradiol. Embryos were treated from 28 to 52 hpf with the chemicals, each at 7 \( \mu \)M, followed by fixation for whole-mount in situ hybridization (Fig. 1A) or quantitative real-time RT-PCR (Fig. 1B) to assess hepcidin RNA levels. We found that genistein treatment was associated with increased intensity of hepcidin expression (Fig. 1A,B) and an expanded domain of expression extending from the liver into the proximal intestine in 100% of 36 embryos, whereas treatment with apigenin, daizdein, estradiol, or genistin did not increase either intensity or extent of expression. However, treatment with the BMP receptor antagonist, dorsomorphin, abrogated genistein’s effect on hepcidin expression (Fig. 1A).

The genistein-induced increase in hepcidin transcript levels was modest (Fig. 1B), related to decreased liver size and only a slight increase in hepatocyte-specific hepcidin expression (Supporting Figs. 1-3). This may explain why, despite the increase in hepcidin expression, staining for hemoglobin (Supporting Fig. 4) did not reveal anemia in any of the genistein-treated embryos (\( n = 50 \)).

**Genistein Increases Hecpidin Expression in HepG2 Cells.** To evaluate whether genistein exerted a conserved effect on human hepatocytes, we tested the compounds in human hepatoma (HepG2) cells. We found that genistein produced a five-fold increase in
Hepcidin expression, as measured by quantitative real-time RT-PCR (Fig. 1C) that was not reproduced by treatment with the other small molecules. The effect was dose dependent (Fig. 1D), with higher increases in Hepcidin expression noted at 20 than 10 μM. In contrast, no significant increase in Hepcidin expression was noted after treatment with estradiol at a broad range of concentrations, ranging from 0.001 to 100 μM (Fig. 1E). The addition of the potent estrogen receptor antagonist, ICI 182,780 (Fig. 1E), failed to
inhibit genistein’s positive effects on Hepcidin transcript levels. In fact, the addition of the estrogen-receptor inhibitor, ICI 182,780, actually increased Hepcidin expression (Fig. 1E), suggesting that inhibition of estrogen receptor signaling may enhance Hepcidin transcript levels.

Genistein May Impair Hepcidin Expression. We considered the possibility that genistein may increase Hepcidin expression by promoting cellular iron loading. To evaluate this hypothesis, we tested the effect of genistein on 55Fe uptake in HepG2 cells. After 24 hours of treatment with genistein, transferrin-bound iron uptake was significantly reduced, compared to vehicle alone (37.27 ± 6.29 versus 67.21 ± 2.755; P < 0.05), whereas nontransferrin-bound iron uptake was not significantly changed from vehicle-treated controls (Fig. 1F). To evaluate effects on hepatocyte iron stores (Supporting Fig. 5), we measured cellular ferritin levels after 24 hours of treatment with genistein or DMSO. We found no significant difference in ferritin levels (0.171 ± 0.058 versus 0.165 ± 0.026; P = 0.92), suggesting that the genistein-induced decrease in transferrin-bound iron uptake is offset by a hepcidin-induced decrease in ferroportin-dependent iron export.

Genistein Increases Hepcidin Promoter Activity. Because genistein did not appear to cause increased Hepcidin expression by enhancing cellular iron uptake, we theorized that it could enhance the effect of either of the major transcription factor pathways that have been implicated in Hepcidin’s regulation: Smad4 or Stat3 signaling. To interrogate these pathways, we transfected HepG2 cells with reporter constructs encoding 3 kb of the human Hepcidin promoter upstream of the firefly luciferase reporter gene. The promoter sequence was either wild type (WT), mutated in the Stat3-binding site, mutated in the Smad4-binding BREs, or mutated in both the Stat3-binding motif and the BREs.33 We found that treatment with either genistein or BMP6 for 6 hours enhanced Hepcidin-luciferase activity by two-fold and three-fold, respectively (Fig. 2A). Mutation of the Smad4-binding BREs, with or without mutation of the Stat3 motif, decreased Hepcidin promoter activity below basal levels in all conditions. However, mutation of the Stat3 motif significantly impaired induction of Hepcidin promoter activity in the genistein-treated, but not the BMP6-treated or vehicle-treated, cells. Thus, the genistein-induced increase in Hepcidin promoter activity required Stat3 as well as Smad4 binding.

Inhibition of Type I BMP Receptor Activity Decreases Genistein’s Effect on Hepcidin Expression. Dorsomorphin is a specific inhibitor of type I BMP receptors that has previously been shown to impair Hepcidin expression.35 Although treatment with dorsomorphin alone greatly reduces Hepcidin expression, as measured by quantitative real-time RT-PCR in HepG2 cells, cotreatment with genistein and dorsomorphin allows the Hepcidin transcript level to remain significantly above the basal level of expression (1.8 ± 0.15 versus 0.99 ± 0.39; P < 0.05; Fig. 2B). These observations imply that genistein activates both BMP-dependent and BMP-independent pathways.

Genistein Treatment Is Associated With Increased Transcript Levels of Many Genes That Are Up-Regulated by BMP6. To compare the effects of genistein and BMP6 on transcription in HepG2 cells, we performed high-throughput RNA-seq and identified the transcripts up-regulated in HepG2 cells after treatment with genistein (10 μM) or BMP6 (50 ng/mL) for 24 hours, in comparison to treatment with vehicle alone. We found that genistein treatment significantly up-regulated 2613 messenger RNA (mRNA) transcripts, whereas BMP6 treatment significantly increased expression of 974 RNA transcripts. Using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, we identified genistein-induced increases in transcripts of genes affecting the ribosome, cell cycle, spliceosome, acute and chronic myeloid leukemia, and the lysosome (Table 1). Among the genes in the leukemic pathway were the transcription factors, Stat3 and TGFβ1, which are known to affect Hepcidin expression.24-27,36 KEGG pathways that were down-regulated after genistein treatment (Supporting Table 2) included those affecting valine, leucine, and isoleucine catabolism, fatty acid metabolism, steroid biosynthesis, and the peroxisome proliferator-activating receptor-signaling pathway.

We found that approximately two thirds of the transcripts that were up-regulated by BMP6 were also up-regulated by genistein, as illustrated in a Venn diagram (Fig. 2C) and heat map (Fig. 2D). Gene Ontology (GO) classification using the DAVID algorithm indicated that genistein and BMP6 both affect 48 fundamental cellular processes (Supporting Table 3), including translation and stress response, whereas KEGG pathway analysis demonstrated that both genistein and BMP6 increase transcript levels of ribosomal proteins (Supporting Table 4).

To validate the results of the RNA-seq analysis, we performed quantitative real-time RT-PCR to detect changes in transcript levels in genes that exhibited increased expression on RNA-seq after genistein treatment (Fig. 3A-E). We found a significant correlation between the natural log of the fold change in gene expression, as measured by quantitative PCR and RNA-seq (R² = 0.7964; Fig. 3A). Genistein
significantly increased transcript levels of Stat3 and the TGFβ1-responsive gene, Serpine1, also known as plasminogen activator inhibitor type 1. In contrast, BMP6 treatment did not induce expression of either Stat3 or Serpine1 (Fig. 3B,C). Genistein treatment produced significant increases in the BMP-regulated transcripts, Id1 and Id3 (1.68 ± 0.11 [P = 0.009] and 2.11 ± 0.21 [P = 0.01], respectively), but these increases (Fig. 3D,E) were far lower than the increases produced by BMP6 (8.4 ± 0.44 [P < 0.001] and 15.3 ± 1.49 [P < 0.001], respectively). As expected, the BMP receptor antagonist, dorsomorphin, repressed transcript levels of Id1 and Id3.

To evaluate genistein’s effect on the Stat3-signaling pathway, in comparison to IL-6 and BMP6, we used quantitative real-time RT-PCR to assess changes in gene expression of Stat3-related genes that were up-regulated by genistein on RNA-seq (Fig. 4A-E). Genistein, BMP6, or IL-6 each produced significantly increased transcript levels of Hepcidin; however, only genistein and IL-6 increased transcript levels of the Stat3 target genes, suppressor of cytokine signaling 3 (SOCS3) and vascular endothelial growth factor A (VEGFA), the Stat2-interacting protein, interferon regulatory factor 9 gene (IRF9), and IL6 receptor alpha. In addition, genistein and IL-6 each up-regulated TGFβ1 (Fig. 4F).

Genistein Increases Stat3 Binding to the Hepcidin Promoter. Because genistein up-regulated several Stat-related genes and mutation of the Stat3-binding
site in the Hepcidin promoter impaired genistein’s effect on Hepcidin promoter activity, we hypothesized that genistein would increase Stat3 binding to the Hepcidin promoter. We performed immunoprecipitation with a Stat3-specific Ab or with nonspecific IgG, followed by quantitative RT-PCR, to amplify the Stat3-binding site. We found that genistein significantly increased Stat3 binding to the Hepcidin promoter (9.2 ± 3.16 versus 0.94 ± 0.11; P = 0.004), whereas BMP6 did not (Fig. 5A).

**Genistein Increases Stat3 Phosphorylation.** To assess the effect of genistein on Stat3 activation in human hepatocytes, we treated HepG2 cells with genistein or IL-6 and assessed the ratio of pStat3 to Stat3 protein from 0 to 60 minutes after the start of treatment (Fig. 5B). We found that genistein significantly increased pStat3/Stat3 over the vehicle control after 60 minutes of treatment (3.65 ± 0.95 versus 1.03 ± 0.21; P = 0.02). As expected, IL-6 also significantly increased pStat3/Stat3 (2.81 ± 0.56 versus 1.00 ± 0.24; P = 0.01), but the effect peaked after 30 minutes of treatment.

**Discussion**

In our study, we identified genistein as the first small-molecule experimental drug to up-regulate Hepcidin transcript levels in vivo and in vitro. Genistein is known to cause both estrogen receptor-dependent and receptor-independent effects. Genistein exhibits estrogenic effects on gene transcription, scavenges free radicals, and inhibits numerous protein tyrosine kinases. We found that genistein’s effect on Hepcidin expression was estrogen receptor independent. Estradiol failed to increase Hepcidin expression in either zebrafish embryos or HepG2 cells, and the potent estrogen receptor inhibitor, ICI 182,780, failed to block genistein’s effect. In fact, we found that ICI 182,780 stimulated Hepcidin expression. These observations agree with recently published data indicating that...
estradiol does not increase Hepcidin expression, and that ICI 182,780 increases Hepcidin transcript levels in HepG2 cells in the presence or absence of estradiol.\textsuperscript{45}

Hepcidin exerts control over iron homeostasis by decreasing intestinal iron absorption and macrophage iron release in response to excessive accumulation of iron in vital organs. Hepatic iron overload increases hepcidin transcript levels in normal mice\textsuperscript{46} and humans.\textsuperscript{46} Thus, we hypothesized that genistein might increase Hepcidin expression by enhancing hepatocyte iron uptake. We found that genistein failed to increase either transferrin-bound or nontransferrin-bound iron uptake. Thus, genistein does not appear to increase cellular iron overload, which is encouraging in a potential therapy for hemochromatosis.

Our \textit{in vivo} zebrafish and human hepatocyte data (Figs. 1 and 2) indicate that BMP signaling is required for hepcidin expression, and that genistein's effect on hepcidin expression is diminished by the BMP antagonist, dorsomorphin. Although our RNA-seq data indicate that genistein up-regulated 68% of the genes that were up-regulated by BMP6, genistein's mode of action differed from BMP6's. Genistein increased Hepcidin promoter activity in a Stat3-dependent manner, whereas BMP6 did not. We also identified several genes in the Janus kinase/Stat-signaling pathway that were up-regulated by genistein, but not by BMP6. For example, Stat3 itself, IL6 receptor, SOCS3, Serpine1, and VEGF\textit{a}, all of which are up-regulated in response to Stat3 activation,\textsuperscript{47} and IRF9, which interacts with Stat2 (reviewed elsewhere\textsuperscript{48}). Increasing IL6 receptor expression would be expected to increase sensitivity to IL6, which, in turn, could promote Hepcidin expression. Genistein also increased the expression of TGF-\textbeta1, a Smad4-signaling protein that has been shown to increase Hepcidin expression.\textsuperscript{36}

Previously, genistein has been reported either to promote or inhibit Stat3 phosphorylation. This apparently paradoxical effect appears to be dose dependent. At lower concentrations, such as those used in our study, genistein promotes Stat3 phosphorylation and cell proliferation,\textsuperscript{49} whereas at high concentrations (40-50
µM) genistein inhibits Stat3 phosphorylation and cell proliferation^{49,50} and promotes apoptosis.^{49} Consistent with these observations, we found that 10 µM of genistein enhanced Stat3 phosphorylation and Stat3 binding to the Hepcidin promoter in cultured human hepatocytes.

We have demonstrated that genistein increases hepcidin expression in human hepatocytes in a Stat3-dependent and Smad4-dependent manner. The screening technique that we have developed may be used to identify other Hepcidin regulatory molecules with different modes of action. Genistein and other candidate molecules may subsequently be tested in preclinical models of iron overload syndromes and used to develop new therapies for iron overload.

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References


