ARTICLE

Increased hepcidin in transferrin-treated thalassemic mice correlates with increased liver BMP2 expression and decreased hepatocyte ERK activation

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ABSTRACT

Iron overload results in significant morbidity and mortality in β-thalassemic patients. Insufficient hepcidin is implicated in parenchymal iron overload in β-thalassemia and approaches to increase hepcidin have therapeutic potential. We have previously shown that exogenous apo-transferrin markedly ameliorates ineffective erythropoiesis and increases hepcidin expression in $Hbb^{th1/th1}$ (thalassemic) mice. We utilize *in vivo* and *in vitro* systems to investigate effects of exogenous apo-transferrin on Smad and ERK1/2 signaling, pathways that participate in hepcidin regulation. Our results demonstrate that apo-transferrin increases hepcidin expression *in vivo* despite decreased circulating and parenchymal iron concentrations and unchanged liver Bmp6 mRNA expression in thalassemic mice. Hepatocytes from apo-transferrin-treated mice demonstrate decreased ERK1/2 pathway and increased serum BMP2 concentration and hepatocyte BMP2 expression. Furthermore, hepatocyte ERK1/2 phosphorylation is enhanced by neutralizing anti-BMP2/4 antibodies and suppressed *in vitro* in a dose-dependent manner by BMP2, resulting in converse effects on hepcidin expression, and hepatocytes treated with MEK/ERK1/2 inhibitor U0126 in combination with BMP2 exhibit an additive increase in hepcidin expression. Lastly, bone marrow erythroferrone expression is normalized in apo-transferrin treated thalassemic mice but increased in apo-transferrin injected wild-type mice. These findings suggest that increased hepcidin expression after exogenous apo-transferrin is in part independent of erythroferrone and support a model in which apo-transferrin treatment in thalassemic mice increases BMP2 expression in the liver and other organs, decreases hepatocellular ERK1/2 activation, and increases nuclear Smad to increase hepcidin expression in hepatocytes.

Introduction

β-thalassemia is characterized by anemia, expanded erythropoiesis, and iron overload with iron overload principally causing morbidity and mortality in these patients.[1] Although iron overload primarily results from transfused erythrocytes, transfusion-independent patients also develop iron overload from increased dietary iron absorption. Iron absorption and iron recycling are regulated by hepcidin, a peptide hormone produced predominantly in the liver. Hepcidin binds fer-

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roportin (FPN1), the iron exporter on enterocytes, hepatocytes, and reticuloendothelial macrophages,[2] and results in FPN1 degradation and decreased release of cellular iron, down-regulating dietary iron absorption, iron release from stores, and tissue iron recycling. Despite iron overload, hepcidin is inappropriately low and is thus implicated as the cause of iron overload in patients with and mouse models of β-thalassemia.[3-7] This lack of appropriate hepcidin response, despite increased parenchymal iron stores, in β-thalassemia suggests a competing hepcidin-suppressing signal.[6-8] In diseases of concurrent iron overload and ineffective erythropoiesis, hepcidin suppression results from secretion of bone marrow factors [(e.g. growth differentiation factor 15 (GDF15), twisted gastrulation 1 (TWSG1), GDF11, and erythroferrone (ERFE)].[9-12] These erythroid regulators of hepcidin and their signaling pathways are active areas of investigation targeted for development of novel therapeutics in iron disorders.

We previously demonstrated that exogenous apo-transferrin (apoTf) in Hbb$^{th1/th1}$ (thalassemic) β-thalassemia intermedia mice markedly ameliorates ineffective erythropoiesis and increases hepcidin expression.[13] Mechanisms of hepcidin regulation involve bone morphogenetic proteins (BMPs). Several BMP signaling molecules up-regulate hepcidin expression *in vitro*[5,14-16] by binding BMP receptors. BMP6 is a principal endogenous BMP regulating hepcidin expression,[16,17] and *Bmp6* knockout mice exhibit hepcidin suppression with iron overload.[17,18] *Bmp6* mRNA is up-regulated in mouse liver following dietary iron overload, suggesting that transcriptional regulation of hepcidin by iron involves an autocrine or paracrine BMP6 effect.[3] However, increased hepcidin in chronically iron-loaded *Bmp6* knockout mice suggests that other pathways stimulate hepcidin expression in response to iron overload.[19] Furthermore, when normalized to liver iron content, Bmp6 expression is not increased in β-thalassemic mice,[5] suggesting that hepcidin regulation in conditions of chronic iron overload, such as β-thalassemia, may involve additional molecules. Other BMPs, including BMP2 and 4, also induce hepcidin regulation *in vitro*[20] and neutralizing BMP2/4 antibodies suppress hepcidin-responsiveness to serum and/or holoTf similar to noggin (BMP receptor blocker) response.[21] Lastly, BMP2 injection results in increased hepcidin expression *in vivo*,[14] but its physiological role in hepcidin regulation has not been fully determined.

Regulation of hepcidin expression in hepatocytes is dependent on the decapentaplegic (Smad) signaling pathway. BMP receptor binding induces phosphorylation of intracellular Smad1/5/8, the association of pSmad1/5/8 with Smad4, and the complex translocation to the nucleus where binding to regulatory elements induces hepcidin expression. Recent evidence suggests that BMP receptor signaling is complex and Smad signaling may integrate with other signaling pathways.[22] Specifically, MAP kinase modulates Smad signaling,[23] and, although the details have not been worked out, may regulate nuclear translocation or transcriptional activity of pSmad1/5/8.[24] Most studies examining such crosstalk used transformed epithelial cell lines, with MEK/ERK1/2 pathway reported to enhance[25] or inhibit[26] Smad activity depending on cell type- or target gene-specificity. Several studies provide indirect evidence that hepatic MEK/ERK1/2 is involved in hepcidin regulation.[21,27,28] In particular, MEK/ERK1/2 inhibition did not suppress hepcidin expression in HepG2 cells[28] despite parallel increases of MEK/ERK1/2 and Smad signaling in response to BMP2 and holoTf. Thus, the physiological relevance of the interactions between these signaling pathways in iron homeostasis is still not completely understood.

We postulate that apoTf systemically affects hepatocyte hepcidin expression *via* the purported "erythroid regulator". In addition, we evaluate the role of addition BMPs in systemic and cellular iron regulation of hepcidin in apoTf-treated mice. Lastly, we hypothesize that MEK/ERK1/2 suppression in hepatocytes is involved in stimulating hepcidin expression in apoTf-treated mice. To understand the mechanisms of hepcidin regulation from these perspectives in apoTf-treated thalassemic mice, we explore iron-related parameters in circulation, in the liver, and in hepatocytes. Our findings demonstrate that reversal of ineffective erythropoiesis and increased hepcidin in apoTf-treated thalassemic mice correlate with decreased hepatocyte MEK/ERK1/2 signaling, increased circulating BMP2, and decreased ERFE expression in erythroid precursors, supporting the hypothesis that exogenous apoTf influences hepcidin expression both *via* erythropoiesis- and iron-related pathways.

Methods

Mice

Hbb$^{th1/th1}$ (thalassemic) mice were backcrossed onto a C57BL6 background, as previously described.[13] Age- and gender-matched 8-10-week old thalassemic and C57BL6 (WT) mice were bred and housed in the animal facility under AAALAC guidelines. The experimental protocols were approved by the Institutional Animal Care and Use Committee. Standard Mouse Chow was used for all experiments (Lab Diet #5001, 270 ppm iron). All mice had access to food and water *ad libitum*.

Transferrin regimen

Mice were treated with 10 mg (400 mg/kg/day) of human apoTf (Kamada, Israel) or same volume of PBS *via* intraperitoneal injections daily for 20 days. This course yielded results consistent with previously published 60 days of injections[13] (*Online Supplementary Figure S1A and B*). Mice were sacrificed on day 3 after the last injection and samples processed for analyses.

Serum parameter analyses

Mouse serum was separated and analyzed using ELISA kits for hepcidin (Hepcidin-Murine Compete™ competitive ELISA, Intrinsic LifeSciences, LLC, La Jolla, CA, USA), BMP2 (Abnova, Taiwan), and the Integra 800 Automated Clinical Analyzer (Roche Diagnostics, IN, USA) for other circulating iron-related parameters.

Non-heme iron spectrophotometry

Iron quantification was performed using the Torrance and Bothwell method.[29] Briefly, desiccated tissue samples were digested in acid-digestion mixture, diluted, and mixed with chromogen reagent. Absorption was measured at 540 nm on a spectrophotometer (Multiskan MCC Microplate Reader, Fisher Scientific).

Primary culture of hepatocytes

Wild-type and thalassemic mouse livers were perfused with PBS followed by Liberase TM (Roche Diagnostics, IN, USA) or filtered collagenase type 1 (Worthington, NJ, USA) using two-step liver perfusion. Live cells were purified by Percoll (Sigma) and plat-

ed, as previously described.[15] Cells were allowed to attach, starved for 18 h, and treated for 24 h with 5% mouse serum with or without 20 μg/mL monoclonal anti-human BMP2/4 antibody (R&D systems). Alternatively, WT serum-treated primary mouse hepatocytes were incubated with increasing doses of MEK/ERK1/2 inhibitor, U0126 [(freshly diluted in DMSO (Promega)] or DMSO (Sigma) 2-2.5 h prior to cell harvest. Lastly, primary mouse hepatocytes were incubated with 5% FBS and increasing doses of BMP2 (Sigma) for 24 h with and without U0126.

Western blot

Liver was homogenized with Protease and Phosphatase Inhibitor Cocktail (Sigma) and total protein extracted. Freshly isolated or cultured hepatocytes were directly lysed by Cell Lysis Buffer (Cell Signaling). Nuclear protein was prepared using NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) as per the manufacturer's instructions. Briefly, samples were homogenized, stained with 0.4% trypan blue, and analyzed by western blot using sub-fraction specific controls. Specifically,

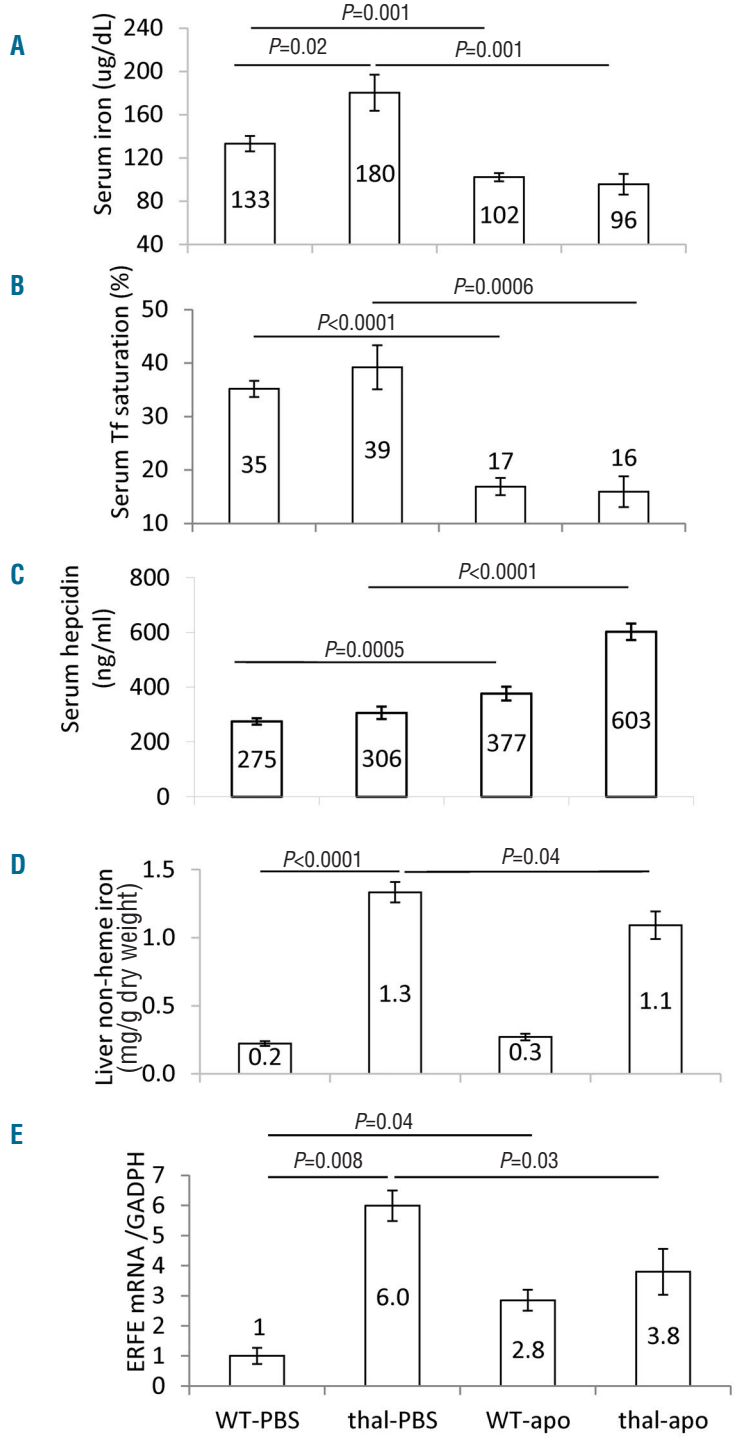

Figure 1. Effect of apo-transferrin injection on iron-related parameters. Serum iron (A) and transferrin saturation (B) (measured in the serum as a ratio of serum iron and total transferrin binding capacity) in PBS-injected WT (n=8), PBS-injected thalassemic (n=6), apoTf-treated WT (n=13), and apoTf-treated thalassemic (n=6) mice. (C) Serum hepcidin concentration measured by ELISA in PBS-injected WT (n=24), PBS-injected thalassemic (n=13), apo-transferrin-treated WT (n=22), and apoTf-treated thalassemic (n=14) mice. (D) Liver non-heme iron concentration measured using spectrophotometry (n=12-14 mice per group). (E) ERFE mRNA expression in sorted bone marrow orthochromatophilic erythroblasts (n=4 sorted samples per group, each sorted sample from 2-3 mice). Tf: transferrin; apo: apo-transferrin; WT: wild type; thal: thalassemic ($Hbb^{th1/th1}$); ERFE: erythroferrone.

membranes were incubated with primary antibodies [Smad1, pSmad1/5/8, Smad4, Ferritin H, ERK1/2, pERK1/2, and TBP (TATA box binding protein) (Cell Signaling)]; Smad7 (Sigma); TGIF, and Lamin B (Santa Cruz); GAPDH and β-actin (Thermo Scientific); and BMP2 (Novus Biologicals)] as well as HRP-conjugated secondary antibodies (Thermo Scientific) and detected using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). The results were quantified using Image J (v1.45q, NIH, USA).

Immunofluorescence

Samples were fixed in 4% paraformaldehyde, washed, permeabilized, blocked, and incubated overnight with primary rabbit anti-pSmad1/5/8 antibody. Control slides were incubated with rabbit IgG. Slides were washed and stained with goat anti-rabbit secondary antibody [(Alexa Fluor® 488 conjugate (Molecular Probes)]. Coverslips were mounted and slides viewed using Zeiss LSM 510 Meta Laser Scanning Confocal microscope.

Fluorescence-activated cell-sorting analysis

Bone marrow cells were processed and analyzed as described previously.[30] Briefly, cells were incubated with anti-CD45 magnetic beads and CD45 negative cells collected, counted, and incubated with anti-mouse TER119-phycoerythrin-Cy7 (PE-Cy7) and CD44-allophycocyanin (APC). Erythroid precursors were identified and sorted using TER119, CD44, and forward scatter on MoFlo® XDP High-Speed Cell Sorter (Beckman Coulter, Miami, FL, USA) using Summit Software (Beckman Coulter, Miami, FL, USA). Post-sort target population purity was confirmed by microscopic morphology evaluation of cytospins.

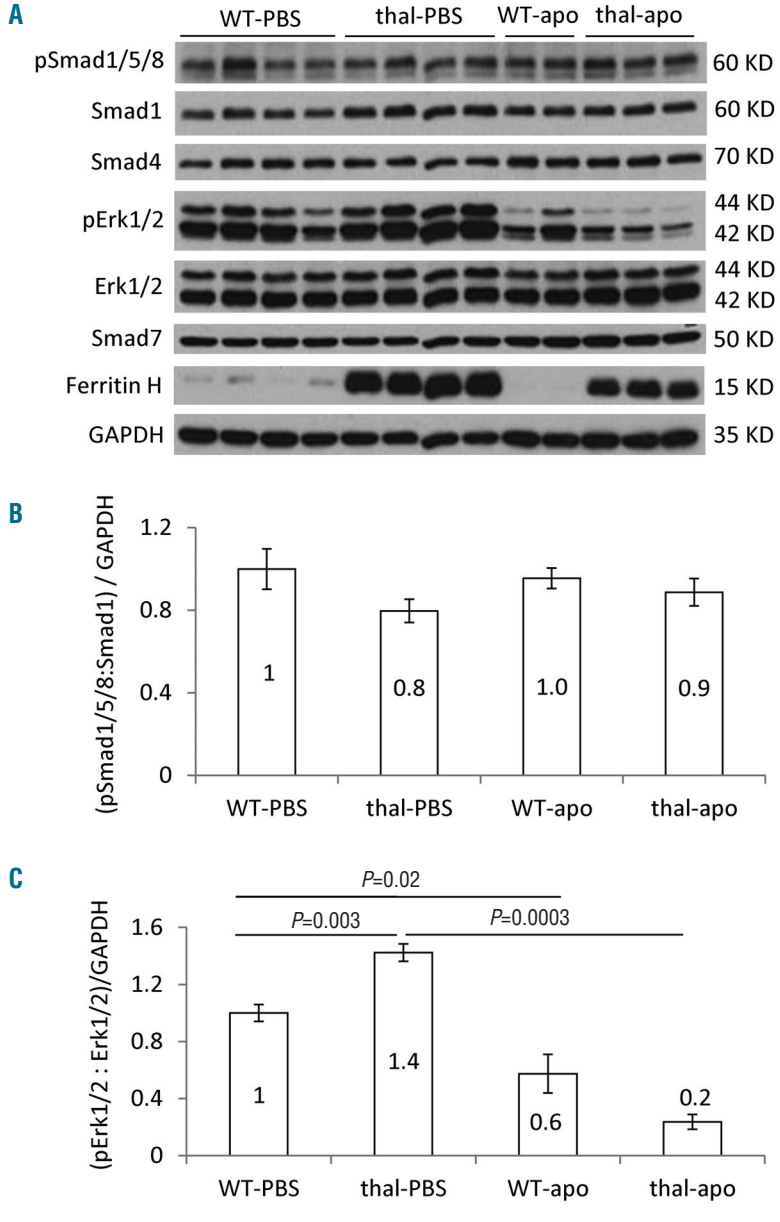

Figure 2. Effect of apo-transferrin treatment on Smad and ERK1/2 signaling to hepcidin in hepatocytes. (A) Phosphorylated Smad1/5/8, Smad1, Smad4, and Smad7, phosphorylated ERK1/2, total ERK1/2, and Ferritin H in fresh hepatocytes detected by western blot (representative gel; n = 6-7 mice per group). (B) Statistical analysis of phosphorylated Smad1/5/8:total Smad1:GAPDH in fresh hepatocytes performed using ImageJ, presented as mean ± SEM (n = 6-7 mice per group). (C) Statistical analysis of phosphorylated ERK1/2:total ERK1/2 relative to GAPDH in fresh hepatocytes performed using ImageJ, presented as mean ± SEM (n=6-7 mice per group). apo: apo-transferrin; WT: wild type; thal: thalassemic ($Hbb^{th1/th1}$).

RNA extraction and quantitative real-time RT-PCR

RNA from hepatocytes or livers was purified using PureLink RNA Mini Kit (Ambion, Life Technology) and analyzed with SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Life Technology). Hepcidin and ERFE mRNA was detected, as previously reported.[12] Primers for mouse BMP6 were designed and confirmed (*Online Supplementary Table S1*). We normalized mRNA concentrations to GAPDH.[8]

Statistical analysis

All data are reported as mean±standard error (SEM). Analysis for statistically significant differences was performed using Student's unpaired *t*-test.

Results

Increased hepcidin and improved iron-related parameters in apoTf-treated thalassemic mice

To evaluate apoTf's effect on iron metabolism, we measured circulating and cellular iron-related parameters in WT, thalassemic, and apoTf-treated WT and thalassemic mice. Serum iron concentration is higher in thalassemic compared to WT mice, and significantly decreases in apoTf-treated mice (Figure 1A). As previously demonstrated,[13] WT and thalassemic mice exhibit similar transferrin saturations which significantly decrease in apoTf-treated mice (Figure 1B and *Online Supplementary Figure S1B*). Serum hepcidin concentration (Figure 1C) and liver Hamp1 mRNA expression (*Online Supplementary Figure S2A*) exhibit similar patterns; although no difference in hepcidin is observed between WT and thalassemic mice, apoTf increases hepcidin expression (Figure 1C and *Online Supplementary Figure S2A*). Furthermore, Id1 mRNA expression is significantly increased in apoTf-treated thalassemic mice (*Online Supplementary Figure S2A*). No differences are observed in other genes relevant to hepcidin regulation (e.g. Tfr2, HFE, HJV, and Tfr1) either between WT and thalassemic mice or between PBS-injected and apoTf-treated thalassemic mice (*Online Supplementary Figure S2B and C*).

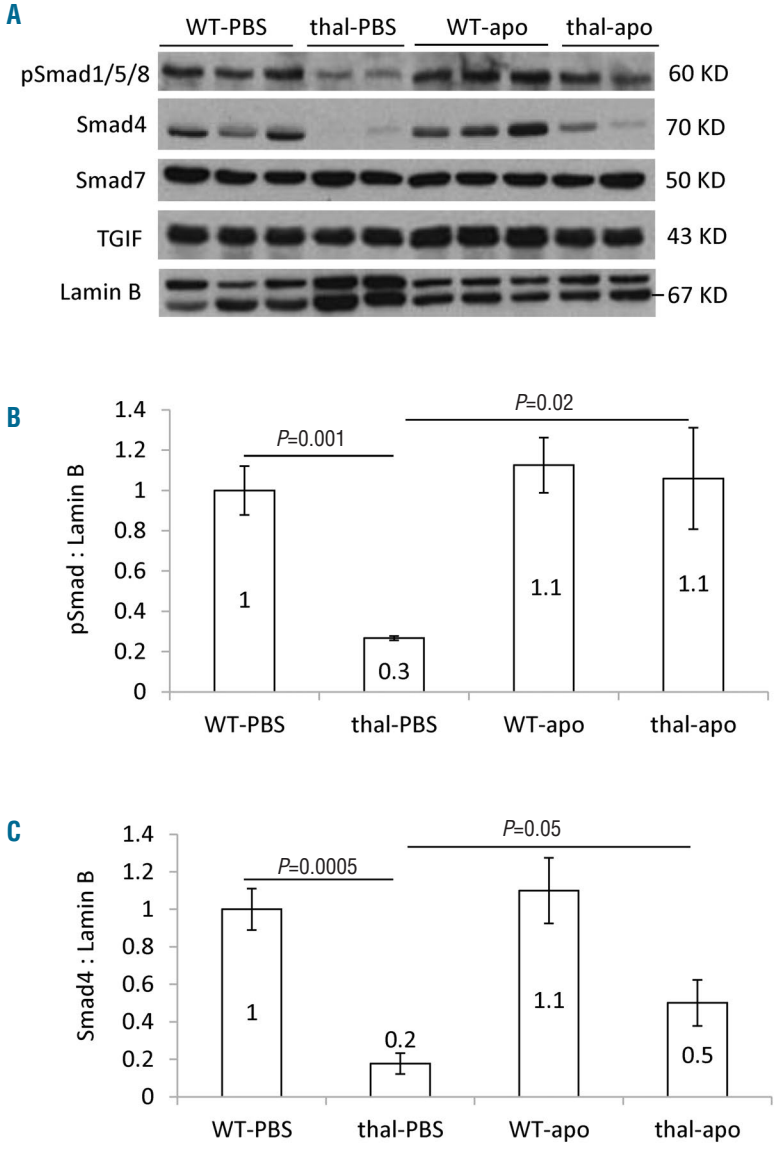

Figure 3. Effect of apo-transferrin injection on nuclear Smad signaling in hepatocytes. (A) Phosphorylated Smad1/5/8, Smad4, Smad7, and TGIF in hepatocyte nuclei detected by western blot (representative gel; n=4-6 mice per group in each experiment). Statistical analysis of phosphorylated Smad1/5/8 (B) and Smad 4 (C) relative to Lamin B (lower band) in hepatocyte nuclei performed using ImageJ, presented as mean ± SEM (n=4-6 mice per group). apo: apo-transferrin; WT: wild type; thal: thalassemic ($Hbb^{th1/th1}$).

Hepcidin concentration is similar in WT and thalassemic mice despite increased liver iron concentration in thalassemic mice (Figure 1D). Importantly, increased hepcidin in apoTf-treated thalassemic mice occurs despite decreased liver iron concentration (Figure 1D), consistent with previously published work on thalassemic mice.[13] ApoTf injections increase hepcidin expression relative to liver iron in both WT and thalassemic mice (*Online Supplementary Figure S2D*). These findings led us to evaluate known iron-responsive regulators of hepcidin expression, including the BMP/SMAD pathway.

Nuclear pSmad1/5/8 and Smad4 increased in apoTf-treated thalassemic hepatocytes

No changes in pSmad1/5/8:Smad1:GAPDH are observed between freshly isolated primary hepatocytes from WT, thalassemic, and apoTf-treated mice (Figure 2A and B). Although thalassemic mice exhibit a greater iron concentration, both in the liver (Figure 1D) and in isolated hepatocytes (Figure 2A), pSmad1/5/8:Smad1:Ferritin H is reduced compared to WT mice and increased by apoTf treatment (*Online Supplementary Figure S2E*). This finding suggests that exogenous apoTf partially restores Smad pathway responsiveness to hepatocellular iron stores and that activation of Smad1/5/8 is inappropriately low in thalassemic mice, consistent with previous findings.[5] No difference in total cellular Smad4 is observed (Figure 2A).

To further assess the Smad pathway, we analyzed positive (Smad 1/5/8 and Smad 4) and negative (TGIF and Smad7) regulatory Smads in hepatocyte nuclear fractions.[31,32] Nuclear pSmad1/5/8 and Smad4 are suppressed in thalassemic mice and increased in apoTf-treated thalassemic mice (Figure 3A-C). Increased hepcidin mRNA expression in apoTf-treated thalassemic mice occurs despite increased Smad7 mRNA expression (*Online Supplementary Figure S2A*) and independent of Smad7 protein concentration as no changes are observed in either total cellular or nuclear fractions (Figures 2A and 3A). TGIF remains unchanged in hepatocyte nuclear fractions from WT, thalassemic, and apoTf-treated thalassemic mice (Figure 3A). Taken together, these findings demonstrate that hepatocellular nuclear pSmad1/5/8 and Smad4 increase in apoTf-treated thalassemic mice despite decreased circulating and tissue iron concentrations.

MEK/ERK1/2 pathway inhibition in vivo and in vitro correlates with increased hepcidin expression

Because MEK/ERK1/2 signaling has been proposed in hepcidin regulation, we investigate pERK1/2 in primary mouse hepatocytes. Both pERK1/2 and pERK1/2:ERK1/2:GAPDH are increased in freshly isolated hepatocytes from thalassemic mice (Figure 2A and C).

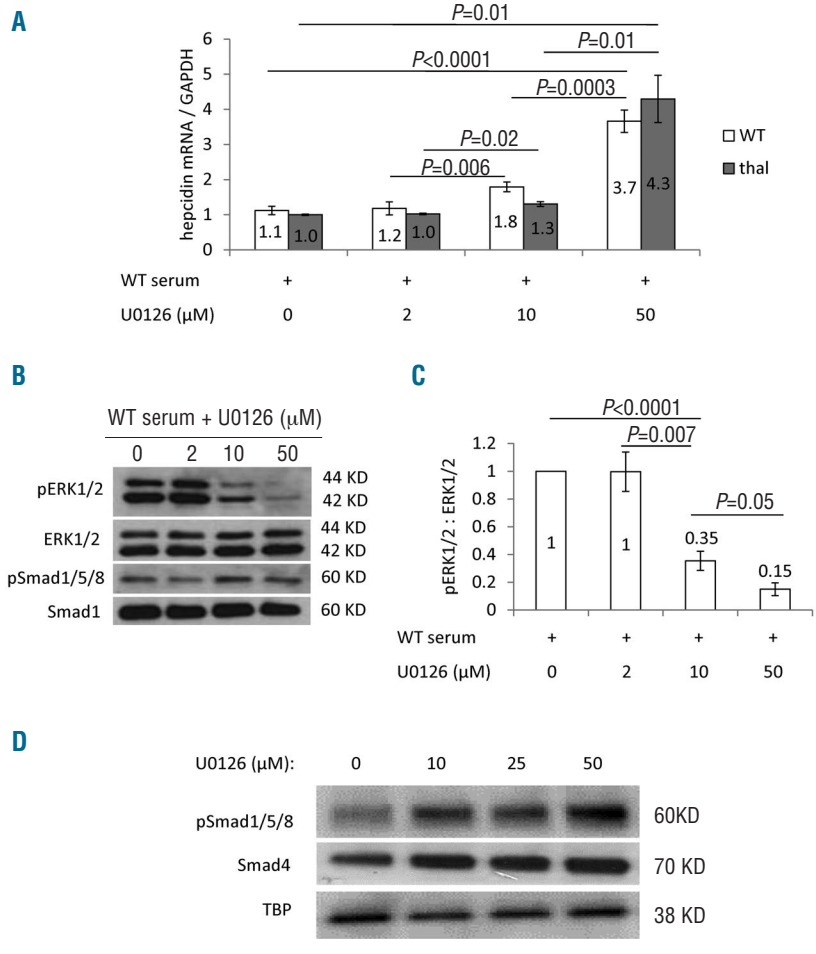

Figure 4. Effects of MEK/ERK1/2 inhibitor U0126 on hepcidin expression *in vitro*. Hepcidin mRNA expression (A) as well as cellular ERK1/2 and Smad1/5/8 signaling (B and C) in primary hepatocytes from WT and thalassemic mice. Cells were cultured with WT mouse serum in the presence of 0, 2, 10, or 50 μM U0126 added 2.5 h prior to cell harvest, normalized to DMSO treated control cells. (D) Nuclear pSmad1/5/8 and Smad4 in WT primary hepatocytes treated with escalating doses of U0126 as above. These *in vitro* results represent 3-6 independent experiments. WT: wild type; TBP: TATA box binding protein.

Both pERK1/2 and pERK1/2:ERK1/2:GAPDH are decreased in apoTf-treated mice (Figure 2A and C), suggesting that increased hepcidin in apoTf-treated mice is a consequence of ERK1/2 pathway suppression. No changes in MEK/ERK1/2 signaling were observed using liver tissue (*data not shown*), consistent with prior results in iron-overloaded mice.[33]

To confirm that ERK1/2 signaling inhibits hepcidin expression, we evaluated serum-treated primary WT and thalassemic hepatocytes and demonstrate a dose-dependent decrease in pERK1/2 and increase in hepcidin expression in response to MEK/ERK1/2 inhibitor, U0126 (Figure 4A-C). U0126 treatment did not change total cellular pSmad1/5/8 (Figure 4B), but nuclear pSmad1/5/8 increased in U0126-treated hepatocytes (Figure 4D), suggesting that increased hepcidin expression in apoTf-treated mice is related to decreased hepatocellular pERK1/2 (Figure 1C and *Online Supplementary Figure S2A*) and functions through increased nuclear pSmad1/5/8 and Smad4.

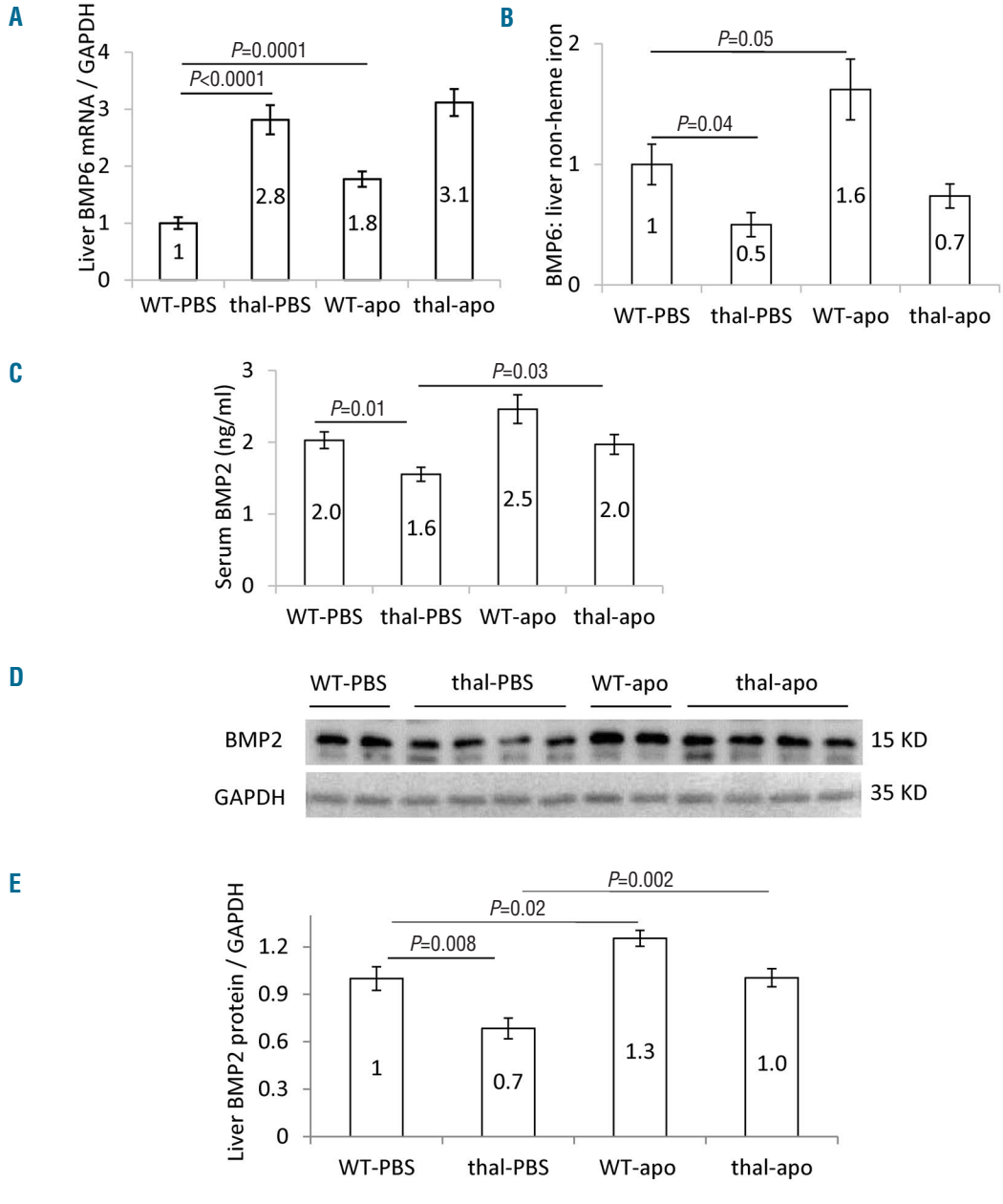

Figure 5. Effect of apo-transferrin treatment on hepcidin regulators BMP2 and BMP6. (A) Liver BMP6 mRNA expression measured by q-RT PCR relative to GAPDH mRNA and normalized to PBS-injected WT mice (n=8-12 mice per group). (B) Statistical analysis of BMP6 mRNA relative to non-heme liver iron, presented as mean ± SEM (n=8-12 mice per group). (C) Serum BMP2 concentration measured by ELISA (n=6-9 mice per group). Western blot (D, representative gel) and statistical analysis (E) of liver BMP2 protein concentration (n=6-9 mice per group). apo: apo-transferrin; WT: wild type; thal: thalassemic ($Hbb^{th1/th1}$).

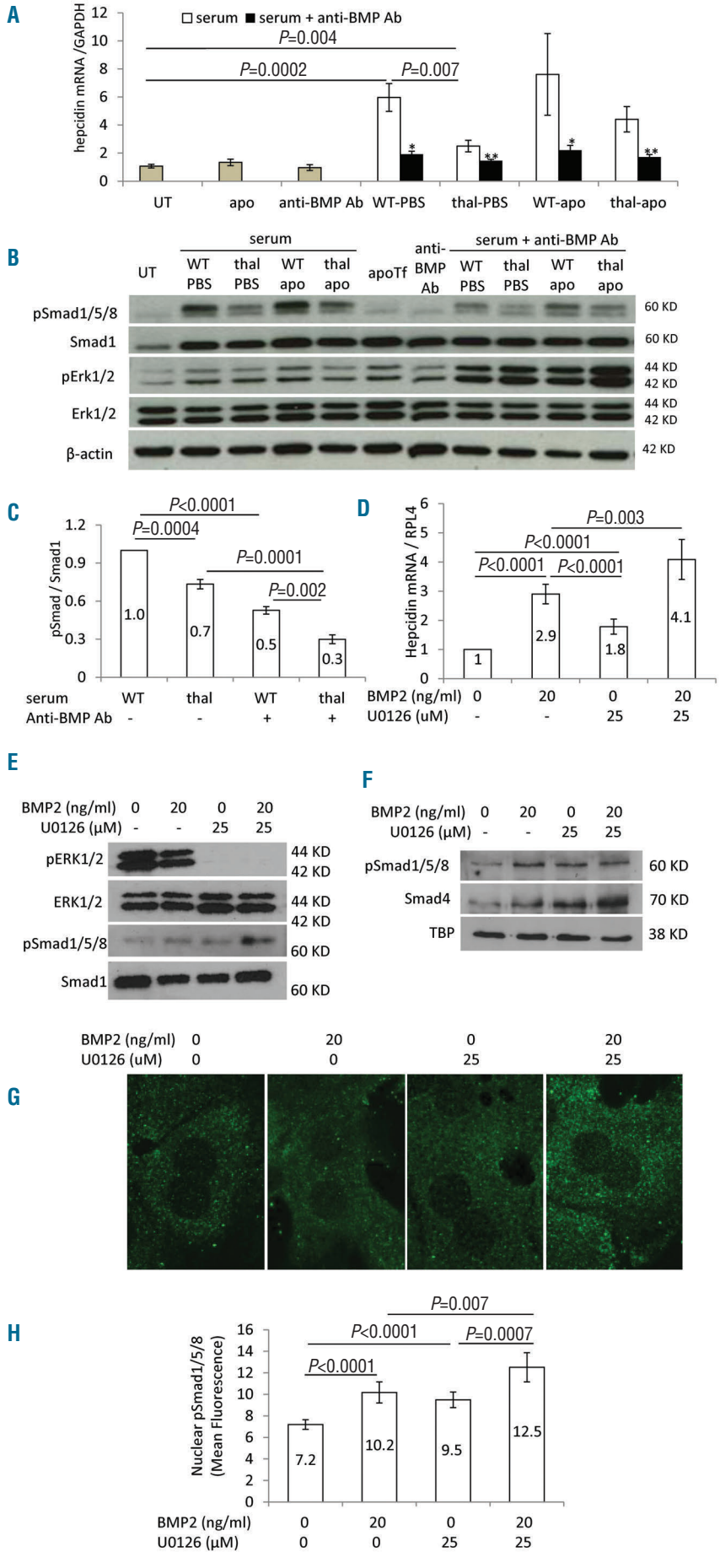

Figure 6. Effects of mouse serum, MEK/ERK1/2 inhibitor U0126, BMP2, and neutralizing BMP2/4 antibody on hepcidin expression *in vitro*. Hepcidin mRNA expression (A) and Smad and ERK1/2 pathway activation (B) in primary WT hepatocytes cultured with different mice sera, with and without the addition of neutralizing anti-BMP2/4 antibodies, and compared with primary cultured hepatocytes directly treated with apo-transferrin. Results are normalized to untreated hepatocytes in culture. Concurrent treatment with serum and neutralizing anti-BMP2/4 antibodies compared with serum or anti-BMP2/4 antibody alone (*P<0.05 and **P<0.004 for each paired condition with and without added anti-BMP2/4 antibody). Gray: no serum. (C) Quantification of phosphorylated relative to total Smad in primary WT hepatocytes treated with WT or thalassemic mouse serum. Hepcidin mRNA (D) as well as ERK1/2 and Smad1/5/8 signaling (E) in WT primary hepatocytes treated with 20 ng/mL BMP2, 25 μM U0126, or the combination. These *in vitro* results represent 6 independent experiments. (F) Nuclear pSmad1/5/8 and Smad4 in WT primary hepatocytes treated with 20 ng/mL BMP2, 25 μM U0126, or the combination as above. These *in vitro* results represent 2 independent experiments. (G) Immunofluorescence using anti-pSmad1/5/8 antibodies in WT primary hepatocytes treated with 20 ng/mL BMP2, 25 μM U0126, or the combination as above. (H) Results were quantified using mean nuclear fluorescence intensity in ImageJ. These *in vitro* results represent 4 independent experiments. UT: untreated; apo: apo-transferrin; WT: wild-type; thal: thalassemic ($Hbb^{th1/th1}$); RPL4: ribosomal protein L4; TBP: TATA box binding protein.

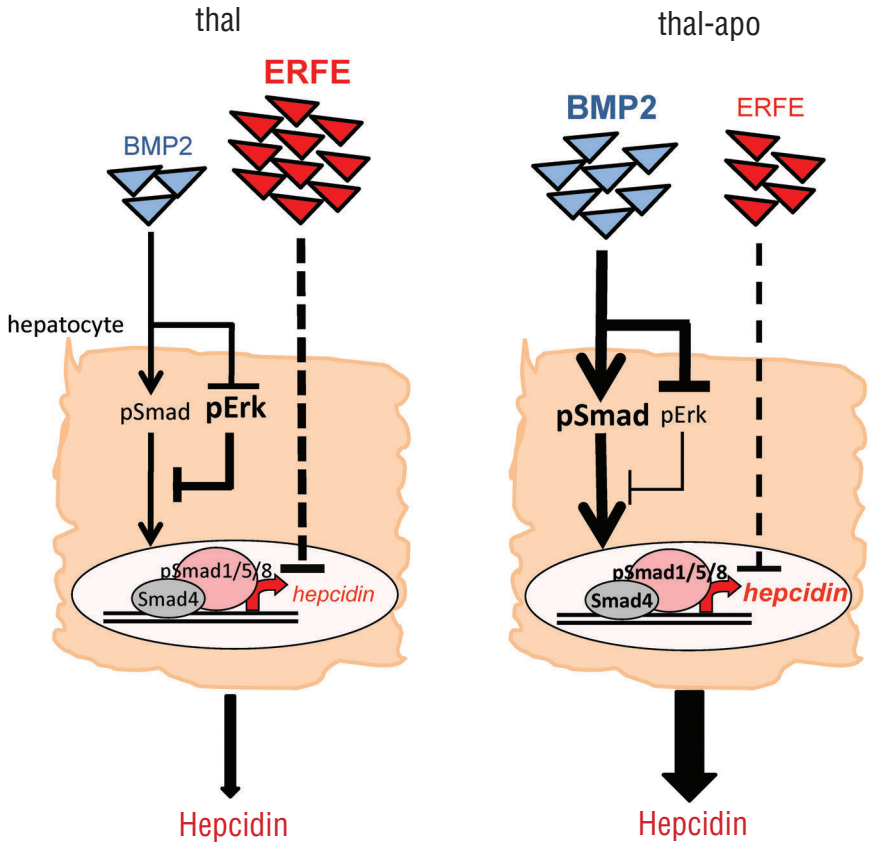

Figure 7. Proposed working model of hepcidin regulation in apo-transferrin-treated thalassemic mice. Apo-transferrin treatment induces circulating BMP2, decreases circulating ERFE, and decreases activation of ERK1/2 in hepatocytes, resulting in increased nuclear Smad signaling and hepcidin expression. ERFE: erythroferrone; thal: thalassemic ($Hbb^{th1/th1}$); apo: apo-transferrin.

BMP2 is associated with increased hepcidin expression in apoTf-treated thalassemic mice

Because BMPs regulate hepcidin *via* Smad signaling, we investigated BMPs in PBS-injected and apoTf-treated mice. We utilized whole liver samples for *Bmp6* mRNA analysis, in the light of evidence that BMP6 induction by dietary iron occurs primarily in liver non-parenchymal cells, rather than hepatocytes.[34] In agreement with this, hepatocytes exposed *in vitro* to mouse serum exhibit unchanged *Bmp6* expression (*data not shown*). Although liver *Bmp6* expression is significantly increased in thalassemic mice (Figure 5A), consistent with higher non-heme liver iron in these mice (Figure 1D), serum hepcidin and liver hepcidin mRNA expression are unchanged from WT mice (Figure 1B and *Online Supplementary Figure S2A*). Liver *Bmp6* expression relative to iron concentration is also suppressed in thalassemic mice (Figure 5B).

However, no significant change in *Bmp6* expression [either absolute (Figure 5A) or relative to liver iron (Figure 5B)] is observed in apoTf-treated thalassemic mice despite a decrease in liver iron (Figure 1D) and increased *Bmp6* expression in apoTf-treated WT mice (Figure 5A and B). We, therefore, evaluated the potential role of other BMPs in hepcidin regulation in apoTf-treated thalassemic mice. Serum and liver BMP2 concentration are lower in thalassemic mice and increased in apoTf-treated thalassemic mice (Figure 5C-E); BMP2 is also increased in apoTf-treated WT mice (Figure 5D and E). BMP2 mRNA and protein expression are undetectable in sorted bone marrow erythroid precursors (*data not shown*), and BMP2 expression in hepatocytes is lower relative to liver with no difference between PBS-injected and apoTf-treated WT and thalassemic mice (*Online Supplementary Figure S3*). No differences in serum BMP4 concentration (*Online Supplementary Figure S4A*) or mRNA expression in the liver or hepatocytes (*Online Supplementary Figure S2B and C*) are observed, and liver as well as bone marrow BMP4 are undetectable by western blot (*data not shown*). These findings suggest that like BMP6, non-parenchymal cells are the main source of BMP2 in the liver, correlating with the increased hepcidin expression in apoTf-treated mice.

To further evaluate the role of BMP2 in apoTf-mediated hepcidin regulation, we analyzed the effect of neutralizing anti-BMP2/4 antibody on serum-treated cultured primary WT hepatocytes. To test the validity of this *in vitro* method, we demonstrate suppressed hepcidin expression and pSmad1/5/8 (Figure 6A-C) with unchanged Smad4 and Smad7 concentrations (*data not shown*) in hepatocytes treated with serum from thalassemic relative to WT mice, providing evidence of a robust culture system.[35] Hepcidin expression is also increased in serum-treated relative to untreated cells (Figure 6A). No differences are observed in apoTf-treated relative to untreated hepatocytes or those treated with serum from apoTf- relative to PBS-treated mice (Figure 6A and B), providing evidence of non-parenchymal cell involvement *via* BMP2 and BMP6 on hepcidin expression. Primary hepatocytes concurrently treated with serum and neutralizing anti-BMP2/4 antibody exhibit suppressed hepcidin expression in each condition relative to serum alone conditions (Figure 6A), again suggesting the importance of extra-cellular BMP2 in hepcidin regulation. A similar pattern of pSmad1/5/8 mRNA and protein suppression is seen in cells exposed to serum with and without anti-BMP2/4 antibody (Figure 6A-C). As

expected, Id1 mRNA expression mimics changes in hepcidin expression (*Online Supplementary Figure S5*). Surprisingly, MEK/ERK1/2 pathway activation is increased in hepatocytes treated by the neutralizing antibody (Figure 6B), suggesting that BMP2 is involved in hepcidin regulation *via* the MEK/ERK1/2 pathway.

To further explore the relationships between BMP-Smad and MEK/ERK1/2 pathways, mouse primary hepatocytes were treated with different doses of BMP2 and U0126. In response to BMP2, we observe dose-dependent increases in hepcidin expression and pSmad1/5/8; and decrease in MEK/ERK1/2 (*Online Supplementary Figure S6A and B*). When primary hepatocytes are treated with BMP2 (20 ng/mL), U0126 (25 μM) or the combination, BMP2 and U0126 each induce hepcidin expression with an additive combined effect (Figure 6D). During these treatments, the ERK1/2 pathway is effectively suppressed by U0126 and by BMP2. Total cellular pSmad1/5/8 is minimally affected by the addition of BMP2 or U0126 individually but induced with the combination of BMP2 and U0126 (Figure 6E). However, BMP2, U0126, and their combination induce nuclear pSmad1/5/8 and especially Smad4 by western blot (Figure 6F) and immunofluorescence (Figure 6G and H). These findings demonstrate that BMP2 signaling and MEK/ERK1/2 suppression each up-regulate hepcidin by increasing nuclear pSMAD1/5/8 and especially Smad4 concentrations in an additive way.

ApoTf-induced hepcidin increase correlates with ERFE suppression only in thalassemic mice

We evaluated candidate "erythroid regulators" in apoTf-treated thalassemic mice. No differences were observed in GDF15 (*data not shown*), TWSG1 (*Online Supplementary Figure S7*), or GDF11 (*Online Supplementary Figure S7*) between bone marrow erythroblasts from WT, thalassemic, and apoTf-treated mice. However, ERFE expression is increased in bone marrow erythroblasts in thalassemic mice and normalized in apoTf-treated thalassemic mice but is surprisingly increased in apoTf-treated WT mice (Figure 1E). These findings support the importance of ERFE as an erythroid regulator in thalassemic mice, suggest that the effect in th1/th1 mice and th3/+ mice[12] is comparable, and provide data consistent with our previously published evidence that apoTf treatment reverses ineffective erythropoiesis in thalassemic mice.[13] Similar results have recently been reported in apoTf-treated th3/+ mice.[36] Lastly, increased hepcidin expression in apoTf-treated WT mice occurs despite an increase in ERFE, suggesting that increased liver BMP2 in apoTf-treated WT and thalassemic mice functions, at least in part, through suppression of MEK/ERK1/2 pathway.

Discussion

Thalassemia provides a model system for investigating the dual and competitive regulation of hepcidin by iron and erythropoiesis. Based on prior observations, we proposed that exogenous apoTf provides a window into the mechanisms responsible for this dual regulation. We found that in apoTf-treated thalassemic mice, hepcidin expression is increased despite a decrease in circulating and parenchymal iron; ERFE expression is decreased in the bone marrow, likely responsible for hepcidin de-repression; increased BMP2 but not BMP6 expression are also involved; and MEK/ERK1/2 pathway activation inversely correlates with hepcidin expression.

As previously reported,[13] apoTf-treated mice did not exhibit any toxicity in our experiments, and exogenous apoTf's effect on iron and erythropoiesis is similar whether administered to older (9-10 months)[13] or younger (8-10 weeks) mice. Furthermore, 20 days of daily apoTf injections result in effects similar (*Online Supplementary Table S2*) to 60 days of injections (*Online Supplementary Figure S1A and B*). ApoTf-treated thalassemic mice exhibit a decrease in systemic iron overload. In addition, hepcidin mRNA expression is again unchanged in thalassemic relative to WT mice and increased in apoTf-treated thalassemic mice.[13] A mouse serum hepcidin ELISA has recently been developed[37] and demonstrates a strong correlation with hepcidin mRNA. Increased hepcidin expression in apoTf-treated thalassemic mice occurs despite decreased serum or liver iron, cytosolic Ferritin H, and circulating transferrin saturation, as previously reported,[13] and thus does not reflect changes in iron status.

Although BMP6 is important in hepcidin regulation, we previously demonstrated that *Bmp6* expression is insufficiently increased relative to liver iron in th3/+ mice.[5] Our current data further demonstrate that liver *Bmp6* mRNA expression is unchanged in apoTf-treated thalassemic mice. Furthermore, *Bmp6* is suppressed relative to liver iron, and although total pSmad1/5/8 is unchanged, nuclear pSmad1/5/8 and Smad4 are suppressed in thalassemic mice, together suggesting that BMP receptor stimulation is dampened and unable to increase nuclear pSmad despite increased Bmp6 expression. Similar findings have been demonstrated in th3/+ mice.[38] Lastly, because liver *Bmp6* mRNA expression is increased in apoTf--treated WT but not thalassemic mice, we hypothesize that BMP6 is involved in hepcidin regulation in response to more acute changes in iron status and not to chronic iron overload in thalassemic mice.

Because other BMPs are involved in Smad1/5/8 activation and hepcidin expression *in vitro*,[15] we explored the role of BMP2 in hepcidin regulation of apoTf-treated thalassemic mice. Our results demonstrate that BMP2 is decreased in the sera and livers of thalassemic mice and increased in sera and livers from apoTf-treated thalassemic mice. These findings are consistent with previously published work on BMP2 expression in human liver tissue and hepatoma-derived cell lines,[20] and non-parenchymal cells in the rat liver.[39] We hypothesize that increased hepcidin in apoTf-treated mice is at least in part due to liver-secreted BMP2 because: 1) BMP2 protein is not detectable in hepatocytes, suggesting that non-parenchymal cells in the liver are involved; 2) non-parenchymal cells in the liver are of mesenchymal origin, the same cells previously implicated in BMP6 secretion;[34] 3) BMP2 secretion has been demonstrated from cells of mesenchymal origin (e.g. vascular and skeletal cells).[40,41] Taken together, these data strongly suggest that non-parenchymal cells of the liver and other organs secrete BMP2 to enable paracrine and endocrine effects on hepcidin regulation in the liver.

A previous publication showed that exogenous BMP2 increases hepcidin expression and lowers serum-iron levels in mice[14] and that BMP inhibitor, noggin, as well as neutralizing anti-BMP2/4 antibodies block hepcidin response to serum and transferrin.[21] The increased BMP2 in human sera activates hepcidin expression *in vitro*, is

blocked by BMP2 immunodepletion,[42] and hepcidin suppression correlates with BMP2 suppression in mice.[43] This previous publication supports the role of BMP2 in apoTf-induced hepcidin upregulation. To further evaluate the effect of BMP2 on hepcidin expression, we treated primary hepatocytes with mouse serum and neutralizing BMP2/4 antibody. Although neutralizing anti-BMP2/4 antibodies suppress hepcidin expression and pSmad1/5/8 as expected, pERK1/2 is increased in neutralizing anti-BMP2/4 antibody-treated hepatocytes relative to serum alone. These findings further support our finding that MEK/ERK1/2 signaling is increased in thalassemic mice and suppressed in apoTf-treated mice.

Transferrin in primary hepatocyte cultures demonstrates complex changes in MEK/ERK1/2 signaling and relationship to Smad signaling. *In vitro*, transient induction of MEK/ERK1/2, Smad1/5/8, and hepcidin expression are observed in serum and/or transferrin-treated primary WT mouse hepatocytes, all blocked by U0126.[21] *In vivo*, neither acute nor chronic iron induced increases in liver pERK1/2 in WT mice,[4] despite associated changes in hepcidin expression. Taken together, these reported observations leave considerable uncertainty regarding the relationship between transferrin, pERK1/2, and hepcidin expression. We demonstrate for the first time increased pERK1/2 in hepatocytes from thalassemic relative to WT mice and decreased pERK1/2 in apoTf-treated thalassemic mice. Hepatocellular pERK1/2 is inversely correlated with hepcidin expression and nuclear Smad in thalassemic mice. In primary hepatocytes, U0126 results in the expected dose-response inhibition of MEK/ERK1/2 and increased hepcidin expression. These *in vitro* experiments reveal, despite unchanged cellular or cytosolic pSmad1/5/8, increased nuclear pSmad1/5/8 and Smad4 when MEK/ERK1/2 is suppressed. In addition, BMP2- and U0126-treated primary mouse hepatocytes reveal an additive increase in hepcidin expression relative to treatment with either agent alone, suggesting a cross-acting function between BMP2-Smad1/5/8 and MEK/ERK1/2 pathways. Supporting these observations, a screen using small-molecule kinase inhibitors found that MEK/ERK1/2 pathway inhibitors increase hepcidin in primary hepatocytes[44] and MEK/ERK1/2 activators decrease BMP-dependent nuclear Smad.[28,44,45] Taken together, our data suggest that activation of hepatocyte MEK/ERK1/2 pathway inhibits hepcidin expression by decreasing nuclear Smad, and that these effects are attenuated by treatment with apoTf.

In thalassemic mice, exogenous apoTf reverses ineffective erythropoiesis and increases hepcidin expression[13] likely by reducing circulating "erythroid regulator" suppression of hepcidin. Prior reports demonstrated that GDF15 does not play a role in erythroid regulation of hepcidin in mice.[38,46] We also analyzed TWSG1 and GDF11 mRNA and did not observe any differences in erythroid precursors between PBS- and apoTf-treated thalassemic mice. However, ERFE mRNA expression is increased in thalassemic mice and normalized in apoTf-treated thalassemic mice. Because ERFE expression is dependent on STAT5 signaling *via* erythropoietin receptor,[12] decreased ERFE expression in apoTf-treated thalassemic mice is likely a consequence of improved erythroid maturation and RBC survival, leading to a decrease in serum erythropoietin and reversal of splenomegaly.[13] This finding confirms the importance of ERFE and its role in the reversal of ineffective erythropoiesis in apoTf-treated thalassemic mice. Furthermore, ERFE expression is increased in apoTf-treated WT mice, consistent with increased erythropoietin[13] and reticulocyte counts (*Online Supplementary Table S2*). Thus, hepcidin expression in apoTf-treated WT mice is increased despite increased ERFE, suggesting that BMP2 provides a dominant effect to increase hepcidin expression and that increased hepcidin expression in apoTf-treated thalassemic mice is a combined effect of increased BMP2 and decreased ERFE. Reagents are currently under development to elucidate ERFE regulation of hepcidin in apoTf-treated thalassemic mice.

We hypothesize that liver and/or serum BMP2 is a previously unexplored upstream suppressor of the MEK/ERK1/2 pathway, inducing hepcidin expression (Figure 7). The mechanism by which this occurs is not yet clear. One possibility is that changes in BMP binding endothelial cell precursor-derived regulator (BMPER) affects BMP signaling. Indeed, the concept of BMPER regulation of hepcidin *via* BMP2 has recently been published.[47] Smad-independent signaling for TGFβ family of ligands, including BMPs, has been proposed.[22] BMP2 exerts its function through both MEK/ERK1/2 and Smad pathways in primary cultured osteoblasts[48,49] and it has been proposed that cells of mesenchymal origin exhibit enhancement while cells of epithelial origin exhibit inhibition of Smad signaling by the MEK/ERK1/2 pathway.[25] The details of a potentially significant cell autonomous cross-talk between the MEK/ERK1/2 and BMP/Smad pathways remain to be elucidated.

Further studies are necessary to explore the potential use of exogenous apoTf to reverse ineffective erythropoiesis in β-thalassemia and other diseases of concurrent anemia and iron overload. Our data present mechanisms for hepcidin de-repression in apoTf-treated thalassemic mice, provide additional therapeutic targets in this pathway, and support our hypothesis that reversal of ineffective erythropoiesis and iron overload require concurrent management in β-thalassemia.

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