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

Huiyong Chen,¹ Tenzin Choesang,¹ Huihui Li,¹ Shuming Sun,¹ Petra Pham,³ Weili Bao,¹ Maria Feola,¹,⁴ Mark Westerman,⁵ Guiyuan Li,² Antonia Follenzi,⁴ Lionel Blanc,⁶ Stefano Rivella,⁷ Robert E. Fleming,⁸ and Yelena Z. Ginzburg¹*

¹Erythropoiesis Laboratory, LFKRI, New York Blood Center, NY, USA; ²Central South University, Changsha, PR China; ³Flow Cytometry Core Laboratory, LFKRI, New York Blood Center, NY, USA; ⁴University of Piemonte Orientale, Amedeo Avogadro, Novara, Italy; ⁵Intrinsic Lifesciences, LLC, La Jolla, CA, USA; ⁶The Feinstein Institute for Medical Research, Manhasset, NY, USA; ⁷Weill Cornell Medical College, NY, USA; and ⁸Saint Louis University, MO, USA

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Correspondence: yginzburg@nybloodcenter.org
# Supplemental Table I: Mouse specific primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-&gt;3’)</th>
<th>Reverse primer (5’-&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP6</td>
<td>CAGCTTGCAAGAAGCATGAG</td>
<td>GGAACACTCTCCATCACAGTAG</td>
</tr>
<tr>
<td>HJV</td>
<td>TATGGGCCAGTCCCCTAGTC</td>
<td>ATCTTGCACTGGGAGTGAGC</td>
</tr>
<tr>
<td>Tfr2</td>
<td>CCTGATCACCCTGCTAATCTTC</td>
<td>TCTTCATCGACCACAAACAC</td>
</tr>
<tr>
<td>HFE</td>
<td>CACCGCGTTCACTTCTCTTA</td>
<td>AAAGAGCTGGTCATCCACATAG</td>
</tr>
<tr>
<td>TWSG1</td>
<td>CACTCTGTGCCAGCGCATGTA</td>
<td>CACACATGCCTCCTTACAG</td>
</tr>
<tr>
<td>BMP2</td>
<td>GACTGCCTGGCTCTCAAAGGTCG</td>
<td>CTGGGGAAGCAGCAACACTA</td>
</tr>
<tr>
<td>BMP4</td>
<td>GAGGAGTTTCCATCACGAAAGAA</td>
<td>ATGCTGCTGAGGTAGAGAG</td>
</tr>
</tbody>
</table>
### Supplemental Table II: RBC parameters in transferrin-treated mice

<table>
<thead>
<tr>
<th></th>
<th>RBCs (x10^6 cells/L)</th>
<th>Hb (g/dL)</th>
<th>MCV (fL)</th>
<th>Retics (x 10^9 cells / L)</th>
<th>CHr (pg)</th>
<th>RDW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - PBS (n=7)</td>
<td>10.5±0.2</td>
<td>14.5±0.3</td>
<td>48±0.3</td>
<td>345±34</td>
<td>15±0.2</td>
<td>13±0.4</td>
</tr>
<tr>
<td>thal - PBS (n=6)</td>
<td>8.5±0.3*</td>
<td>7.1±0.3*</td>
<td>36±1.1*</td>
<td>2514±186*</td>
<td>12±0.2*</td>
<td>34±0.7*</td>
</tr>
<tr>
<td>WT - apo (n=8)</td>
<td>10.5±0.4</td>
<td>10.6±0.6**</td>
<td>41±0.3**</td>
<td>374±21</td>
<td>12±0.1**</td>
<td>15±0.4**</td>
</tr>
<tr>
<td>thal - apo (n=6)</td>
<td>13.3±0.5***</td>
<td>10±0.4***</td>
<td>29±0.7***</td>
<td>907±141***</td>
<td>10±0.1***</td>
<td>25±0.8***</td>
</tr>
</tbody>
</table>

Data represented as mean ± s.e.m. *P<0.0002 thal-PBS versus WT-PBS; **P<0.003 WT-apo versus thal-apo; ***P<0.0002 thal-apo versus thal-PBS; WT = wild type; thal = thalassemic (Hbb^th1/th1); apo = apo-transferrin; RBCs = red blood cells; Hb = hemoglobin; MCV = mean corpuscular volume; Retics = reticulocyte count; CHr = corpuscular hemoglobin of reticulocytes; RDW = red cell distribution width.
SUPPLEMENTAL METHODS

**In vivo experiments** All 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 or 60 days as described previously. Blood and tissues were processed for analyses. Erythroid precursors were collected from bone marrow as reported. Serum BMP4 was measured using ELISA kits (US Biological, MA).

**Ex vivo experiments** Primary hepatocytes were isolated using two-step liver perfusion and cultured as previously described. After 12 hours of starvation, cells were treated for 24 hours with 5% mouse serum or combined with 20 μg/mL monoclonal anti-human BMP2/4 antibody (R&D systems), or incubated with 5% FBS and 0, 5, 10, 20 ng/ml BMP2 (Sigma) for 24 hours. After treatments, cells were harvested for RNA and protein analyses.

**Quantitative real-time RT-PCR** Total RNA were extracted using PureLink RNA Mini Kit (Ambion, Life Technology) and analyzed with SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Life Technology). Primers of HFE, HJV, TfR1, TfR2, TWG1, BMP2, and BMP4 were shown in Supplemental Table I. Smad7 and GDF11 mRNA levels were detected using primers as previously reported. mRNA concentrations were normalized to GAPDH.

**Western Blot** Proteins were separated on 10% SDS-polyacrylamide gels by electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were processed and incubated with primary antibodies (Smad1, pSmad1/5/8, Ferritin H, ERK1/2, and pERK1/2 (Cell Signaling); GAPDH (Thermo Scientific)) as well as HRP-conjugated secondary antibodies (Thermo Scientific).
SUPPLEMENTAL REFERENCES


**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure 1:** Comparison of 20 and 60 days of apo-transferrin injections in thalassemic mice. (A) Spleen size (measured as a ratio of spleen to body weight) and (B) transferrin saturation (measured in the serum as a ratio of serum iron to total transferrin binding capacity). n=5-18 mice per group (Tf = transferrin; apo = apo-transferrin; WT = wild type; thal = thalassemic (Hbb<sup>th1/th1</sup>))

**Supplemental Figure 2:** Effect of apo-transferrin injection on hepcidin, Id1, and Smad7 expression, other genes related to hepcidin regulation in the liver and Smad phosphorylation in hepatocytes. Liver hepcidin, Id1, and Smad7 (A); Tfr2, HFE and HJV (B); as well as Tfr1 (C) mRNA expression relative to GAPDH and normalized to PBS-injected WT mice (n = 8-12 per group). (D) Hepcidin mRNA normalized to non-heme iron (n = 8-12 per group). (E) Statistical analysis of phosphorylated Smad1/5/8 relative to Smad1 normalized to Ferritin H in primary hepatocytes performed using ImageJ, presented as mean ± SEM (n = 6-7 mice per group). (apo = apo-transferrin; WT = wild type; thal = thalassemic (Hbb<sup>th1/th1</sup>))

**Supplemental Figure 3:** BMP2 mRNA expression in the liver and hepatocytes. Values shown are the means ± SEM of −ΔCt values (Ct GAPDH − Ct hepcidin). (n = 4-6 mice per group; apo = apo-transferrin; WT = wild type; thal = thalassemic (Hbb<sup>th1/th1</sup>))
**Supplemental Figure 4:** Effect of apo-transferrin treatment on BMP4 serum concentration and mRNA expression in the liver and hepatocytes. (A) Serum BMP4 concentration measured by ELISA (n = 6-9 mice per group). Liver (B) and hepatocyte (C) BMP4 mRNA expression relative to GAPDH normalized to PBS-injected WT mice (n = 8-12 mice per group). (WT = wild type; thal = thalassemic (Hbb<sup>th1/th1</sup>); apo = apo-transferrin)

**Supplemental Figure 5:** Id1 mRNA expression in primary hepatocytes in vitro. Primary WT hepatocytes treated with serum from WT or thalassemic mice after PBS or apo-transferrin injection, normalized to untreated hepatocytes in culture. Concurrent treatment with serum and neutralizing anti-BMP2/4 antibodies compared with primary hepatocytes treated with serum or anti-BMP2/4 antibody alone. These in vitro results represent 4-6 independent experiments. (* P=0.04 serum alone vs. serum + Ab) (UT = untreated; apo = apo-transferrin; WT = wild type; thal = thalassemic (Hbb<sup>th1/th1</sup>); Ab = anti-BMP2/4 neutralizing antibody)

**Supplemental Figure 6:** Effects of BMP2 on hepcidin expression as well as Smad1/5/8 and ERK1/2 signaling in vitro. Hepcidin mRNA expression (A) as well as ERK1/2 and Smad1/5/8 signaling (B) in WT mouse primary hepatocytes treated with 0, 5, 10, and 20 ng/ml BMP2. These results represent 3 independent experiments.

**Supplemental Figure 7:** Effect of apo-transferrin treatment on bone marrow GDF11 expression. TWSG1 (A) and GDF11 (B) in sorted bone marrow orthochromatophilic erythroblasts (n = 4 sorted samples per group, each sorted sample from 2-3 mice). (WT = wild type; thal = thalassemic (Hbb<sup>th1/th1</sup>); apo = apo-transferrin; TWSG1 = twisted gastrulation factor 1; GDF11 = growth differentiation factor 11)
Supplemental Figure 1

A

![Bar graph showing spleen/body weight with transferrin saturation (%).](image)

- **WT 0d**: 0.020
- **20d**: 0.008
- **60d**: 0.007

- **WT**: n=18
- **thal - apo**: n=14
- **n=8**: n=5

B

![Bar graph showing transferrin saturation (%).](image)

- **WT**: 34
- **0d**: 32
- **20d**: 21
- **60d**: 24

- **WT**: n=5
- **thal - apo**: n=14
- **n=8**: n=10

Supplemental Figure 2

D

![Graph showing hepcidin mRNA: liver non-heme iron comparison.]

- WT-PBS: 1.0
- thal-PBS: 0.2
- WT-apo: 1.6
- thal-apo: 0.5

Significance levels:
- P=0.001
- P=0.03
- P=0.007

E

![Graph showing pSmad 1/5/8 / Smad1: Ferritin H comparison.]

- WT-PBS: 1.0
- thal-PBS: 0.04
- WT-apo: 1.7
- thal-apo: 0.07

Significance levels:
- P=0.02
- P=0.005
- P=0.005
Supplemental Figure 3

-ΔCt (BMP2-GAPDH)

liver  | hepatocytes

WT-PBS  |  thal-PBS  |  WT-apo  |  thal-apo

-8.5  |  -8.7  |  -8.9  |  -8.3
-12.8  |  -11.8  |  -12.6  |  -12.1
Supplemental Figure 4

A

Serum BMP4 (ng/ml)

WT-PBS  thal-PBS  WT-apo  thal-apo

B

Liver BMP4 mRNA / GAPDH

WT-PBS  thal-PBS  WT-apo  thal-apo

C

Hepatocyte BMP4 mRNA / GAPDH

WT-PBS  thal-PBS  WT-apo  thal-apo

P=0.07
Supplemental Figure 5

ID1 mRNA / GAPDH

- UT
- Ab BMP
- WT-PBS
- thal-PBS
- WT-apo
- thal-apo

P=0.002
P=0.008
P=0.09

serum
serum + Ab
Supplemental Figure 6

A

![Bar graph showing the effect of BMP2 on hepcidin mRNA/GAPDH levels.](image)

BMP2 (ng/ml) 0 5 10 20

- BMP2 (ng/ml)
- hepcidin mRNA/GAPDH
- P<0.0001
- P=0.002
- P=0.05

pSmad1/5/8

- Smad1
- pERK1/2
- ERK1/2

- pSmad1/5/8
- Smad1
- pERK1/2
- ERK1/2

- 60 KD
- 60 KD
- 44 KD
- 44 KD
- 42 KD
- 42 KD

- BMP2 (ng/ml)
- 0 5 10 20

- BMP2 (ng/ml) 0 5 10 20