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1 **RESEARCH ARTICLE**

2

3 **Atoh8: a transcriptional regulator of *HAMP* is down-regulated in HuH7 cells**
4 **cultured in the β -thalassemia patient sera and β -thalassemic mice**

5

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18 **KEY WORDS:** Atoh8, *HAMP*, Thalassemia, Iron overload, erythropoiesis

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1 **ABSTRACT**

2 **Objective:** This study aimed to demonstrate the down-regulation of Atoh8 relating to hepcidin
3 suppression in β -thalassemia. **Methods:** HuH7 cells were cultured in DMEM medium
4 supplemented with 20% β -thalassemia patient sera (HbE/ β -thalassemia and β -thalassemia major)
5 or normal serum as a control. To investigate the effect of Atoh8 on *HAMP* transcription in β -
6 thalassemia, HEK293 cells were transfected with ATOH8-FLAG expression plasmid and cultured
7 in DMEM medium supplemented with 20% β -thalassemia patient sera. *Atoh8* and *HAMP* mRNA
8 expression were analyzed by qPCR, and *HAMP* promoter activity was detected by the luciferase
9 reporter assay. In addition, hepatic *Atoh8* and *Hamp1* mRNA expression in β -thalassemic mice
10 were also determined. **Results:** In the cultured HuH7 cells, *HAMP* mRNA levels and *HAMP*
11 promoter activity in all β -thalassemia serum groups were significantly lower than the normal serum
12 group. Interestingly, our study found that Atoh8 expression was down-regulated in the same
13 direction as *HAMP* expression being suppressed despite iron overload. The Atoh8 transfection
14 could enhance *HAMP* expression in endogenous *HAMP* mRNA levels as well as *HAMP* promoter
15 activity in HEK293 cells cultured in the β -thalassemia patient sera. In accordance with this, hepatic
16 *Atoh8* and *Hamp1* mRNA expression in β -thalassemic mice were also down-regulated.
17 **Conclusion:** It's likely that the increased erythropoietic activity in β -thalassemia would suppress
18 Atoh8 expression, subsequently down-regulating hepcidin expression. Therefore, Atoh8 would be
19 a novel regulator of *HAMP* transcription in the β -thalassemia.

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18 Thailand for supplying the research equipment.

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1 **1. Introduction**

2 Hepcidin, an iron regulatory hormone, is a 25-amino acid peptide produced by hepatocytes
3 in the liver (1-6). It plays a critical role in the balance of systemic iron homeostasis by inducing
4 the internalization and degradation of the iron- exporter ferroportin on enterocytes, hepatocytes
5 and macrophages (7-11). Thus the increase of hepcidin inhibits duodenal iron absorption and iron
6 release from hepatocytes and macrophages. This can limit iron overload; however, urinary and
7 serum hepcidin levels in β -thalassemia are reduced (12-15) leading to increased iron absorption
8 which exacerbates iron overload condition.

9 Hepatic hepcidin (*HAMP*) mRNA expression is regulated in by three major stimuli including
10 iron levels, inflammation and erythropoietic activity via different signal transduction pathways.
11 The regulation of *HAMP* transcription responds to changes of tissue iron through the bone
12 morphogenic protein (BMP) signaling pathway and a change of hepatic BMP6 levels (16, 17). The
13 *HAMP* transcription is activated by the inflammation via STAT-3 signaling pathway (18). The
14 signal transduction pathway to regulate *HAMP* transcription under the erythropoietic activity
15 remains unclear. Two molecules produced by erythroid precursors, the growth differentiation
16 factor 15 (GDF15) and the twisted gastrulation factor 1 (TWSG1), and the bone morphogenetic
17 protein binding endothelial cell precursor-derived regulator (BMPER) produced by endothelial
18 cells have been reported as the negative regulator of *HAMP* transcription by BMP signaling
19 pathway (19-22). In addition, a recent study suggested that erythroferrone (ERFE), produced by
20 erythroid precursors, acts as a new erythroid-derived hormone and inhibits hepcidin expression
21 during stress erythropoiesis (23). In β -thalassemia studies, Tanno et al demonstrated that GDF15
22 levels were elevated in β -thalassemia patient serum with low hepcidin and that GDF15 suppressed
23 hepcidin mRNA expression in human hepatocyte cultures (19). In contrast, Casanovas et al

1 reported that the hepcidin expression was suppressed in *Gdf-15^{-/-}* mice subjected to phlebotomy
2 and that bone marrow GDF15 were not increased in the β -thalassemic mice (24). Therefore, the
3 relationship between GDF15 and hepcidin regulation remains controversial. TWSG1 interfered
4 with BMP-mediated hepcidin expression in human hepatocyte cultures and was up-regulated in
5 the β -thalassemic mice (20). ERFE mRNA expression was increased in the β -thalassemic mice,
6 and hepcidin suppression in ERFE-deficient mice was failed after hemorrhage (23). However,
7 human correlative studies of TWSG1 and ERFE have not been investigated yet.

8 Recently, Patel et al (25) has identified *Atoh8*, atonal homolog 8 (*Drosophila*), as a novel
9 positive regulator of *HAMP* transcription via two independent mechanisms: E-box dependent
10 transcriptional activation; and the increase of cellular pSMAD1,5,8 levels in BMP signaling
11 pathway. In addition, the down-regulation of *Atoh8* was observed in mice with increased
12 erythropoietic activity, which appears to override the activation of *Atoh8* by the increased iron
13 levels. All of these explain how hepcidin suppression was carried on by increased erythropoietic
14 activity in the conditions of liver iron loading and increased BMP6 levels without any change in
15 pSMAD1,5,8 levels (26). Given that the increased erythropoiesis activity suppressed *Atoh8*
16 expression similar to hepcidin expression we hypothesized that *Atoh8* may be a novel candidate
17 transcriptional regulator of *HAMP* in the β -thalassemia.

18

19 **2. Materials and Methods**

20 ***2.1. β -Thalassemia serum samples***

21 10-18 years old male (N=11) and female (N=12) HbE/ β -thalassemia patients (HbE/T-Male
22 and HbE/T-Female) and male (N=9) and female (N=6) β -thalassemia major patients (TM-Male
23 and TM-Female) from Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand were the

1 volunteers for this research. The β -thalassemia patients are usually treated with iron chelator(s)
2 (DFP, DFO or DFX) and regularly receive blood transfusion. The experimental protocol was
3 conducted with the approval of the Research Ethics Committee, Faculty of Medicine, Chiang Mai
4 University, Thailand (Research ID: 2151/ Study Code: BIO-2556-02151). Their bloods were
5 collected monthly for 3 months before they receive the blood transfusion. The bloods were
6 collected into 5-ml vacuum tubes, left to clot at room temperature for 30 minutes and centrifuged
7 at 3,000g for 10 minutes. The sera were collected and stored at -20°C until use. All sera in the same
8 group were pooled together and blood iron parameters were determined. Serum Iron (SI) and Total
9 Iron Binding Capacity (TIBC) were analyzed by Clinical Chemistry Laboratory, Maharaj Nakorn
10 Chiang Mai Hospital, Chiang Mai, Thailand. Non-transferrin bound iron (NTBI) concentrations
11 were measured by using NTA chelation/HPLC method (27). Serum ferritin levels were analyzed
12 by Tumor Marker Laboratory, Faculty of Medicine, Chiang Mai University, Thailand. Serum
13 hepcidin levels were estimated by using Hepcidin (Hepc) ELISA Kit (CEB979Hu, Uscon Life
14 Science Inc., Hubei, China).

15 ***2.2. Cell culture and cell transfection***

16 ***2.2.1. Cell culture***

17 Human hepatoma cell lines (HuH7) or human embryonic kidney 293 cell lines (HEK293) were
18 cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-
19 glutamine and 1% penicillin-streptomycin and incubated at 37°C under a humidified 5% CO_2
20 atmospheric condition.

21 The conditions for culturing cells in human sera were determined to imitate β -thalassemia
22 patients as previously described (28). 24-hour (60% confluent) grown cells were starved in serum-
23 free DMEM medium supplemented with 1% penicillin-streptomycin for 24 hours as a starvation

1 step. Subsequently, the medium was removed and the cells were grown in DMEM medium
2 supplemented with 20% human sera [Normal (Invitrogen, UK), male HbE/ β -thalassemia, female
3 HbE/ β -thalassemia, male β -thalassemia major and female β -thalassemia major], 2 mM L-
4 glutamine and 1% penicillin-streptomycin for 24 hours. *HAMP* and *Atoh8* mRNA expression were
5 analyzed by qPCR.

6 **2.2.2. Cell transfection**

7 **2.2.2.1. *HAMP* promoter transfection in HuH7 cells**

8 To confirm endogenous *HAMP* mRNA levels from qPCR result, the *HAMP* promoter assay
9 was performed. Before the cells were grown in DMEM medium supplemented with human sera,
10 *HAMP* reporter plasmid was co-transfected with a TK-renilla control reporter plasmid at a 1:4 ratio
11 in serum-free DMEM medium supplemented with 1% penicillin-streptomycin into the HuH7 cells
12 for 24 hours in the starvation step as mentioned above by using Fugene 6 (Promaga, UK) according
13 to the manufacturer's instructions (29). After the transfected cells were grown in DMEM medium
14 supplemented with human sera, *HAMP* promoter activity was detected by luciferase reporter assay.

15 **2.2.2.2. *Atoh8* and *HAMP* promoter transfection in HEK293 cells**

16 To study the effect of *Atoh8* on *HAMP* transcription, HEK293 cells, known as the model for
17 *Atoh8* transfection as the previous study (25), were transfected with ATOH8-FLAG expression
18 plasmid or empty plasmid (Origen vector) in serum-free DMEM medium supplemented with 1%
19 penicillin-streptomycin for 6 hours in the starvation step as mentioned above by using Fugene 6.
20 After the transfected cells were grown in DMEM medium supplemented with human sera, *Atoh8*
21 mRNA and *HAMP* mRNA expression were determined by qPCR.

22 For *HAMP* promoter assay, *HAMP* reporter plasmid was co-transfected with a TK-renilla
23 control reporter plasmid (1:4 ratio) and ATOH8-FLAG expression plasmid in serum-free DMEM

1 medium supplemented with 1% penicillin-streptomycin into the HEK293 cells for 6 hours in the
2 starvation step by using Fugene 6 (25). After the transfected cells were grown in DMEM medium
3 supplemented with human sera, *HAMP* promoter activity was detected by the luciferase reporter
4 assay.

5 **2.3. Animals**

6 7-8 months old male (N=5) and female (N=5) C57/BL6, wild type (WT, $\mu\beta^{+/+}$) and
7 heterozygous β -globin knockout (BKO, $\mu\beta^{\text{th-3/+}}$), were kindly supplied by Thalassemia Research
8 Center, Institute of Molecular Bioscience, Mahidol University, Salaya Campus, Thailand (30, 31)
9 and conducted under the Animal Ethical Committee of Medical Faculty, Chiang Mai University,
10 Thailand (Reference Number 42/2556). The mice were sacrificed and their livers were collected
11 to determine hepcidin1 (*Hamp1*) and *Atoh8* mRNA expression by qPCR.

12 **2.4. RNA extraction and cDNA synthesis**

13 RNA was extracted from HuH7 cells or HEK293 cells by using TRIzol reagent (Invitrogen,
14 UK). In mice, RNA was extracted from 100 mg of liver by using TRIzol and the genomic DNA
15 was removed by using Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare,
16 Buckinghamshire, UK) according to the manufacturer's protocol. 1 μg of RNA was reverse
17 transcribed into complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription
18 Kit (Applied Biosystems, Foster City, USA).

19 **2.5. Quantitative real-time PCR (qPCR)**

20 **2.5.1. In cell culture**

21 The qPCR reactions of cDNA were performed using TaqMan Gene Expression Assay
22 (Applied Biosystems) on the ABI PRISM 7900 HT PCR machine (Applied Biosystems, Paisley,
23 UK) or ABI 7500 fast real-time PCR machine (Applied Biosystems, Paisley, UK) according to the

1 manufacturer's protocol. mRNA expression of *HAMP* (Hs00221783_m1) or *Atoh8*
2 (Hs01031629_m1) was normalized to housekeeping gene *RLP19* (Hs01577060_gH) and
3 expressed as a fold change by using $\Delta\Delta$ CT method (32).

4 **2.5.2. In mice**

5 The qPCR reactions of cDNA were performed using EXPRESS SYBR® GreenER™ qPCR
6 Supermix Universal Kits (Invitrogen, Carlsbad, CA, USA) on the ABI 7500 real-time PCR
7 machine (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. The
8 primer sequences used in qPCR are presented as follows; *mHamp1* forward:
9 CCTGAGCAGCACCTATC, *mHamp1* reverse: TGCAACAGATACCACACTGGG,
10 *mAtoh8* forward: TCAGCTTCTCCGAGTGTGTG, *mAtoh8* reverse:
11 TAGCCTGTGGCAGGTCCTACT (25), *mActb* forward: GGTCCACACCCGCCAC, *mActb* reverse:
12 GTCCTTCTGACCCATTCCCA. mRNA expression of *Hamp1* or *Atoh8* was normalized to
13 housekeeping gene β -actin (*Actb*) and expressed as a fold change by using $\Delta\Delta$ CT method (32).

14 **2.6. Luciferase reporter assays**

15 The luminescence from HuH7 cells or HEK293 cells was detected by using Dual-Luciferase
16 Reporter Assay according to the manufacturer's instructions (Promega, UK) and measured by a
17 luminometer (Promega, UK). Relative luciferase activity was calculated from the ratio of the signal
18 of firefly luciferase to TK-renilla luciferase and expressed as the fold change to the control group.

19 **2.7. Statistical Analysis**

20 The data are presented as means \pm SD. Data analysis was undertaken by using SPSS program,
21 and statistical significance was determined by using one-way analysis of variance (ANOVA). The
22 p value <0.05 was considered significant.

23

1 **3. Results**

2 **3.1. Blood iron parameters**

3 The blood iron parameters of the pooled human sera: normal, male HbE/ β -thalassemia,
4 female HbE/ β -thalassemia, male β -thalassemia major and female β -thalassemia major were
5 analyzed as shown in the **Table 1**. Serum iron, transferrin saturation, plasma NTBI and plasma
6 ferritin levels in all β -thalassemia patient sera were higher than those in normal serum although
7 plasma hepcidin levels were not different, suggesting that the thalassemia patient sera have iron
8 overload condition.

9 **3.2. Effect of β -thalassemia patient sera on *HAMP* and *Atoh8* expression in HuH7 cells**

10 After 24-hour (60% confluent) grown HuH7 cells were starved in serum-free DMEM
11 medium for 24 hours, HuH7 cells were cultured in DMEM medium supplemented with 20%
12 human sera: normal, male HbE/ β -thalassemia, female HbE/ β -thalassemia, male β -thalassemia
13 major and female β -thalassemia major for 24 hours. Subsequently, *HAMP* and *Atoh8* mRNA
14 expression were detected by qPCR. *HAMP* (**Figure 1A**) and *Atoh8* (**Figure 1B**) mRNA levels and
15 *HAMP* promoter activity (**Figure 2**) in all β -thalassemia serum groups were significantly lower
16 than those in the normal serum group, and there was no difference between genders. Interestingly,
17 our study demonstrated that β -thalassemia patients have not only the decreased *HAMP* expression
18 but also the reduced *Atoh8* expression.

19 **3.3. Hepatic *Hamp1* and *Atoh8* mRNA expression in β -thalassemic mice**

20 Hepatic *Hamp1* (**Figure 3A**) and *Atoh8* (**Figure 3B**) mRNA levels in BKO mice were lower
21 than those in WT mice but significantly in female. However, the *Hamp1* and *Atoh8* mRNA
22 expression were not different between genders.

1 **3.4. Effect of *Atoh8* transfection on *HAMP* expression in HEK293 cells grown in β -**
2 ***thalassemia* patient sera**

3 To determine whether *Atoh8* regulates *HAMP* transcription in β -thalassemia, HEK293 cells
4 were transfected with increasing amounts of the ATOH8-FLAG expression plasmid and grown in
5 the β -thalassemia patient sera. The HEK293 cells were transfected with 0.5, 1, 2 and 4 μ g ATOH8-
6 FLAG expression plasmid and grown in DMEM medium containing 20% female HbE/ β -
7 thalassemia serum for 24 hours. The *Atoh8* transfection could significantly induce *Atoh8* mRNA
8 expression (**Figure 4A**) and up-regulate *HAMP* mRNA expression (**Figure 4B**) in the dose-
9 dependent manner at 2 and 4 μ g ATOH8-FLAG expression plasmid. In accordance with
10 endogenous *HAMP* mRNA levels, *HAMP* promoter activity was more sensitive and significantly
11 increased in the dose-dependent manner with 0.5, 1 and 2 μ g ATOH8-FLAG expression plasmid
12 (**Figure 5**). Furthermore, the *Atoh8* transfection could significantly and markedly enhance *HAMP*
13 expression in both endogenous *HAMP* mRNA and *HAMP* promoter activity not only in the female
14 HbE/ β -thalassemia serum group but also in normal, male HbE/ β -thalassemia, male β -thalassemia
15 major and female β -thalassemia major serum group (**Figure 6 and 7**). The empty plasmid (Origen
16 vector) did not effect on *Atoh8* (**Figure 6A**) and *HAMP* (**Figure 6B**) mRNA levels as well as
17 *HAMP* promoter activity (**Figure 7A**) when compared to the untransfected ATOH8-FLAG group.
18 Thus the *Atoh8* transfection could up-regulate the expression of *Atoh8* probably subsequently
19 activating *HAMP* expression.

20

21 **4. Discussion**

22 The down-regulation of hepcidin expression in β -thalassemic mice (33-38) and the low
23 hepcidin levels in β -thalassemia patients (12-15, 39) have been previously reported. Although our

1 study showed the indifference of hepcidin levels between β -thalassemia patient serum and normal
2 serum as the previous studies (15, 40, 41), HepG2 grown in the medium containing the thalassemia
3 sera revealed the down-regulation of hepcidin expression (28, 40). Interestingly, this study
4 demonstrated that not only hepcidin but also *Atoh8* was down-regulated in β -thalassemia. The
5 decrease of *Atoh8* mRNA levels also related to the reduction of *HAMP* expression, endogenous
6 *HAMP* mRNA and *HAMP* promoter activity, in the HuH7 cells cultured in DMEM medium
7 supplemented with 20% β -thalassemia patient sera for 24 hours. In this condition, erythropoietic
8 regulatory proteins still effect on *HAMP* expression as same as in the patients. According to the
9 suppression of hepatic *Atoh8* and hepcidin expression in the mice under the conditions with an
10 increased erythropoiesis activity (e.g. hemolytic anemia, hypoxia, erythropoietin treatment, and
11 hypotransferrinaemia) (21, 25, 42-46), this study also showed the lower hepatic *Atoh8* mRNA
12 levels related to the decreased hepatic *Hamp1* mRNA levels in β -thalassemic mice. Courselaud
13 and colleagues demonstrated that hepcidin expression varied between genders of mice (47), but
14 not in some study (41) as well as our data *Atoh8* and hepcidin expression in the β -thalassemia
15 patient serum groups and in the β -thalassemic mice were not different between genders. In
16 addition to erythropoietic activity, *Atoh8* expression responded to a change of iron levels. Hepatic
17 *Atoh8* and *Hamp1* mRNA levels were increased in mice fed with an iron supplemented-diet and
18 in mice treated with holo-transferrin, whereas those were decreased in mice fed with an iron
19 deficient-diet (25, 48). Conversely, *Atoh8* and *HAMP* mRNA expression were suppressed in HuH7
20 cells cultured in the medium containing the β -thalassemia patient sera even though their serum
21 iron and transferrin saturation levels are very high. Similarly, in hypotransferrinaemic (HPX) mice
22 with high degree of chronic anemia and liver iron overload, hepatic *Atoh8* and *Hamp1* expression
23 were also down-regulated (21, 25). *Atoh8* mRNA expression was also inhibited in *Hamp1*^{-/-} mice

1 after PHZ treatment although their serum iron was still high (25, 44). As previously shown, it is
2 likely that the up-regulation of Atoh8 by iron was overridden by the increased erythropoiesis
3 activity similar to the regulation of hepcidin. Therefore, in β -thalassemia Atoh8 expression was
4 suppressed by the increased erythropoiesis activity in a similar manner and direction to hepcidin
5 expression.

6 Furthermore, Atoh8 expression was also suppressed in *Hamp1*^{-/-} mice after PHZ treatment
7 increasing erythropoiesis activity, suggesting that Atoh8 is an upstream regulator of *Hamp1* (25).
8 In HEK293 cells, Atoh8 could stimulate *HAMP* transcription in two ways, directly via E-boxes
9 and indirectly through increased pSMAD1,5,8 levels (25). In this study, Atoh8 transfection in
10 HEK293 cultured in the medium containing the β -thalassemia patient sera could induce the *HAMP*
11 expression in both endogenous mRNA and promoter activity. It suggested that the down-regulation
12 of Atoh8 expression in β -thalassemia resulted from the increased erythropoietic activity and
13 subsequently led to the decrease of *HAMP* expression. Therefore, in β -thalassemia Atoh8 may
14 be a novel transcriptional regulator of *HAMP*, which is responsive to the erythropoietic activity.
15 This is a new research for therapies and management in β -thalassemia patients.

16

17 **Conflict of Interests**

18 The authors attest to having no conflicting interests.

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33 mouse liver. *Blood*. 2008;112(4):1503-9.

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1 **Table 1** Blood iron parameters of human sera

Blood chemistry	Normal	HbE/T- Male	HbE/T- Female	TM- Male	TM- Female
SI (µg/dl)	52	140	135	161	155
TIBC (µg/dl)	339	148	135	161	155
Transferrin saturation (%)	15.34	94.59	100	100	100
NTBI (µM)	-0.39	7.31	7.77	5.46	4.07
Ferritin (ng/ml)	26.31	1307	1812	2000	1405
Hepcidin (ng/ml)	34.69	42.78	38.08	32.90	47.76

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Figure legends

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Figure 1 Effect of β -thalassemia patient sera on *HAMP* and *Atoh8* mRNA expression in HuH7 cells grown in DMEM medium supplemented with 20% human sera for 24 hours. Relative *HAMP* (A) and *Atoh8* (B) mRNA expression from qPCR analysis were acquired by normalizing *HAMP* mRNA or *Atoh8* mRNA to *RPL19* mRNA. The samples were determined in quadruplicate in two independent experiments. Values are expressed as mean \pm SD derived from a single experiment with four biological replicates for the fold change as compared to the normal serum group. The experiment shown is representative of two similar experiments. *p<0.05 when compared with the normal serum group.

Figure 2 Effect of β -thalassemia patient sera on *HAMP* promoter activity in HuH7 cells co-transfected with *HAMP* reporter plasmid and TK-renilla and grown in DMEM medium supplemented with 20% human sera for 24 hours. Promoter activity was expressed as the relative luciferase activity to the normal serum group. The samples were determined in triplicate. Values are expressed as mean \pm SD for the fold change as compared to the normal serum group. *p<0.05 when compared with the normal serum group.

Figure 3 Hepatic *Hamp1* and *Atoh8* mRNA expression of male (N=5) and female (N=5) mice. Relative *Hamp1* (A) and *Atoh8* (B) mRNA expression in WT and BKO mice from qPCR analysis were acquired by normalizing *Hamp1* mRNA or *Atoh8* mRNA to *Actb* mRNA. Values are expressed as mean \pm SD for the fold change as compared to the WT-male group.

Figure 4 *Atoh8* and *HAMP* mRNA expression in HEK293 cells transfected with (1, 2 and 4 μ g) ATOH8-FLAG expression plasmid and grown in DMEM medium supplemented with 20% female HbE/ β -thalassemia serum for 24 hours. Relative *Atoh8* (A) and *HAMP* (B) mRNA expression from qPCR analysis were acquired by normalizing *Atoh8* mRNA or *HAMP*

1 mRNA to *RPL19* mRNA. The samples were determined in triplicate. Values are expressed as
2 mean±SD for the fold change as compared to the untransfected ATOH8-FLAG group. *p<0.05
3 when compared with the untransfected ATOH8-FLAG group.

4 **Figure 5 *HAMP* promoter activity in HEK293 cells co-transfected with *HAMP* reporter**
5 **plasmid, TK-renilla and (0.5, 1 and 2 µg) ATOH8-FLAG expression plasmid and grown in**
6 **DMEM medium supplemented with 20% female HbE/ β-thalassemia serum for 24 hours.**

7 Promoter activity was expressed as the relative luciferase activity to the untransfected ATOH8-
8 FLAG group. The samples were determined in triplicate. Values are expressed as mean±SD for
9 the fold change as compared to the untransfected ATOH8-FLAG group. *p<0.05 when compared
10 with the untransfected ATOH8-FLAG group.

11 **Figure 6 *Atoh8* and *HAMP* mRNA expression in HEK293 cells transfected with (2 µg)**
12 **ATOH8-FLAG expression plasmid or empty plasmid (Origen vector) and grown in DMEM**
13 **medium supplemented with 20% human sera for 24 hours. Relative *Atoh8* (A) and *HAMP***

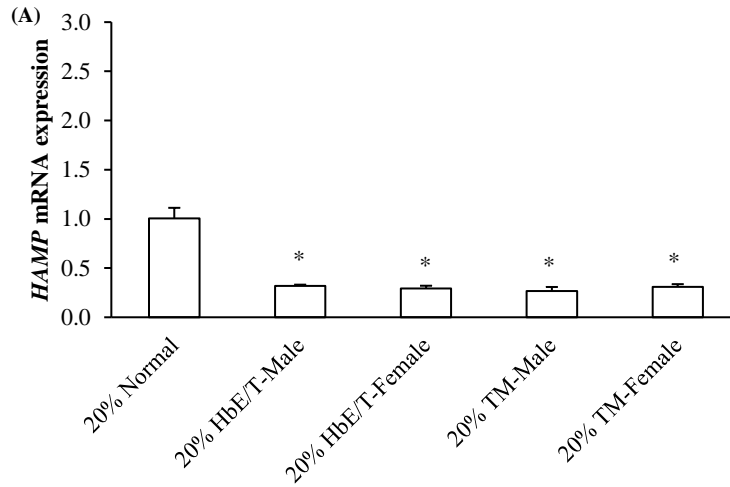
14 (B) mRNA expression from qPCR analysis were acquired by normalizing *Atoh8* mRNA or *HAMP*
15 mRNA to *RPL19* mRNA. The samples were determined in triplicate. Values are expressed as
16 mean±SD for the fold change as compared to the untransfected ATOH8-FLAG group.

17 **Figure 7 *HAMP* promoter activity in HEK293 cells co-transfected with *HAMP* reporter**
18 **plasmid, TK-renilla and (0.5 and 1 µg) ATOH8-FLAG expression plasmid (or Origen**
19 **vector) and grown in DMEM medium supplemented with 20% human sera (A: Female**

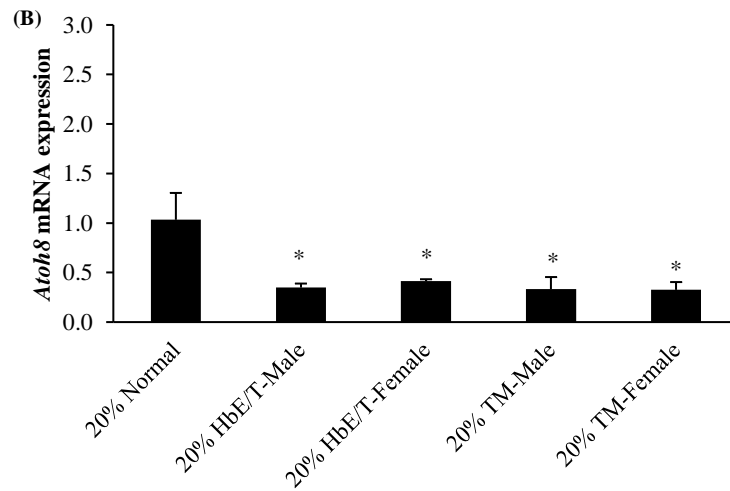
20 **HbE/β-thalassemia, B: Normal, C: Male HbE/β-thalassemia, D: Male β-thalassemia major,**
21 **D: Female β-thalassemia major) for 24 hours. Promoter activity was expressed as the relative**
22 **luciferase activity to the untransfected ATOH8-FLAG group. The samples were determined in**

- 1 triplicate. Values are expressed as mean \pm SD for the fold change as compared to the untransfected
- 2 ATOH8-FLAG group. *p<0.05 when compared with the untransfected ATOH8-FLAG group.
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4 **Figure 1 Effect of β -thalassemia patient sera on *HAMP* and *Atoh8* mRNA expression in HuH7**

5 **cells grown in DMEM medium supplemented with 20% human sera for 24 hours. Relative**

6 *HAMP* (A) and *Atoh8* (B) mRNA expression from qPCR analysis were acquired by normalizing

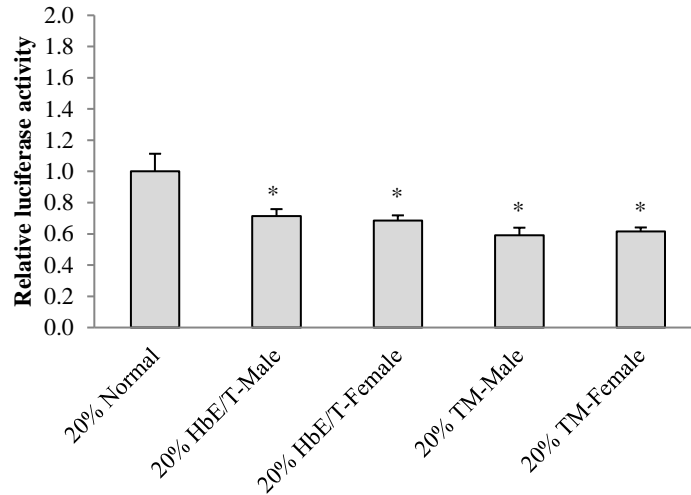
7 *HAMP* mRNA or *Atoh8* mRNA to *RPL19* mRNA. The samples were determined in quadruplicate

8 in two independent experiments. Values are expressed as mean \pm SD derived from a single

9 experiment with four biological replicates for the fold change as compared to the normal serum

10 group. The experiment shown is representative of two similar experiments. *p<0.05 when

11 compared with the normal serum group.

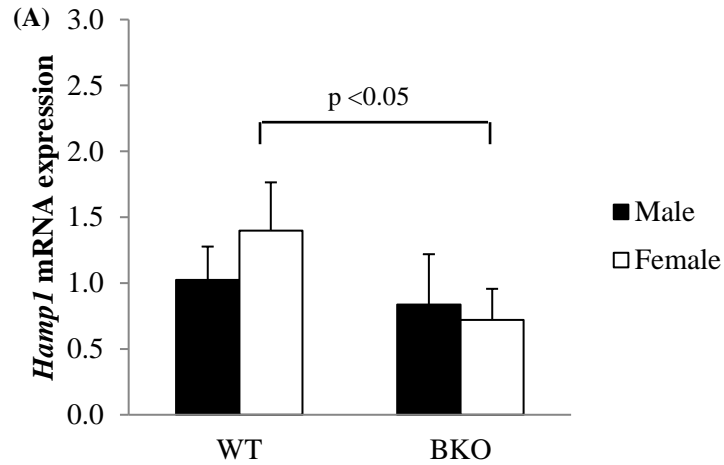


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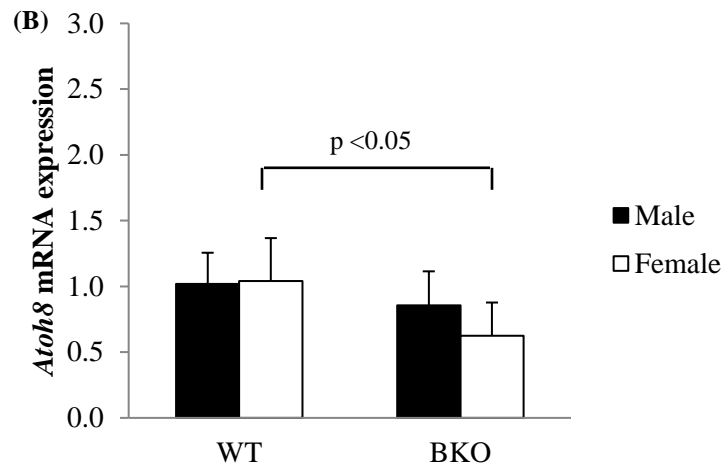
2 **Figure 2 Effect of β -thalassemia patient sera on *HAMP* promoter activity in HuH7 cells co-**
 3 **transfected with *HAMP* reporter plasmid and TK-renilla and grown in DMEM medium**
 4 **supplemented with 20% human sera for 24 hours. Promoter activity was expressed as the**
 5 **relative luciferase activity to the normal serum group. The samples were determined in triplicate.**
 6 **Values are expressed as mean \pm SD for the fold change as compared to the normal serum group.**

7 *p<0.05 when compared with the normal serum group.

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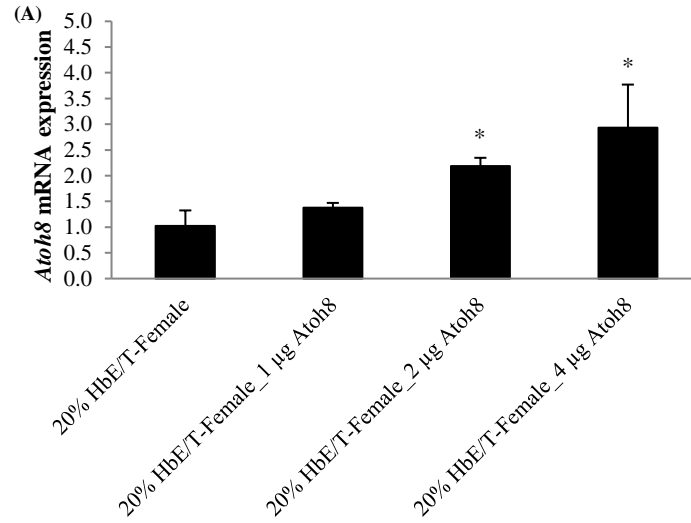
3 **Figure 3 Hepatic *Hamp1* and *Atoh8* mRNA expression of male (N=5) and female (N=5) mice.**

4 Relative *Hamp1* (A) and *Atoh8* (B) mRNA expression in WT and BKO mice from qPCR analysis

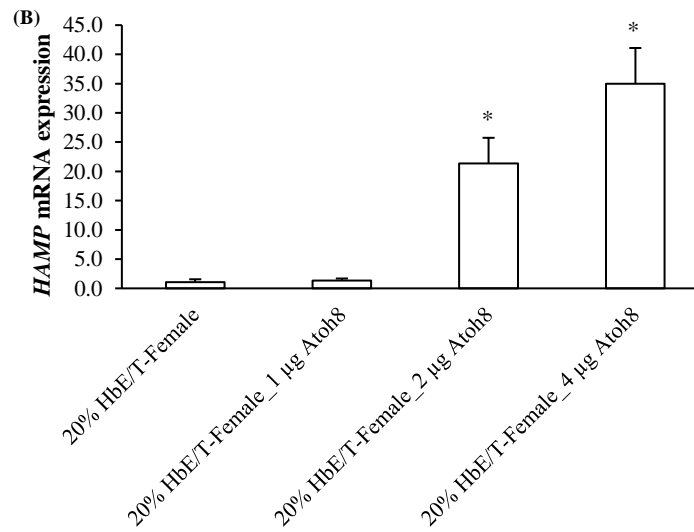
5 were acquired by normalizing *Hamp1* mRNA or *Atoh8* mRNA to *Actb* mRNA. Values are expressed

6 as mean \pm SD for the fold change as compared to the WT-male group.

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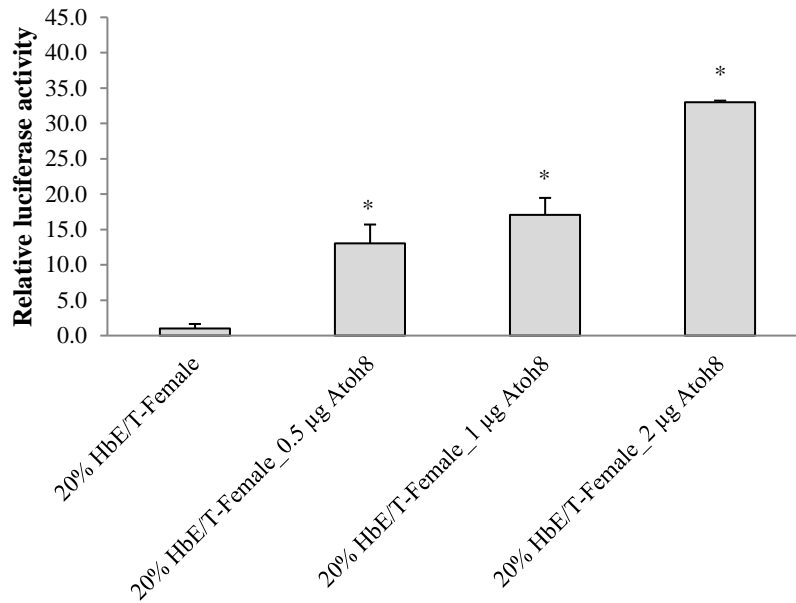


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3 **Figure 4** *Atoh8* and *HAMP* mRNA expression in HEK293 cells transfected with (1, 2 and 4
 4 **µg)** ATOH8-FLAG expression plasmid and grown in DMEM medium supplemented with
 5 **20% female HbE/ β-thalassemia serum for 24 hours.** Relative *Atoh8* (A) and *HAMP* (B)
 6 mRNA expression from qPCR analysis were acquired by normalizing *Atoh8* mRNA or *HAMP*
 7 mRNA to *RPL19* mRNA. The samples were determined in triplicate. Values are expressed as
 8 mean±SD for the fold change as compared to the untransfected ATOH8-FLAG group. *p<0.05
 9 when compared with the untransfected ATOH8-FLAG group.



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2 **Figure 5** *HAMP* promoter activity in HEK293 cells co-transfected with *HAMP* reporter

3 plasmid, TK-renilla and (0.5, 1 and 2 µg) ATOH8-FLAG expression plasmid and grown in

4 DMEM medium supplemented with 20% female HbE/ β-thalassemia serum for 24 hours.

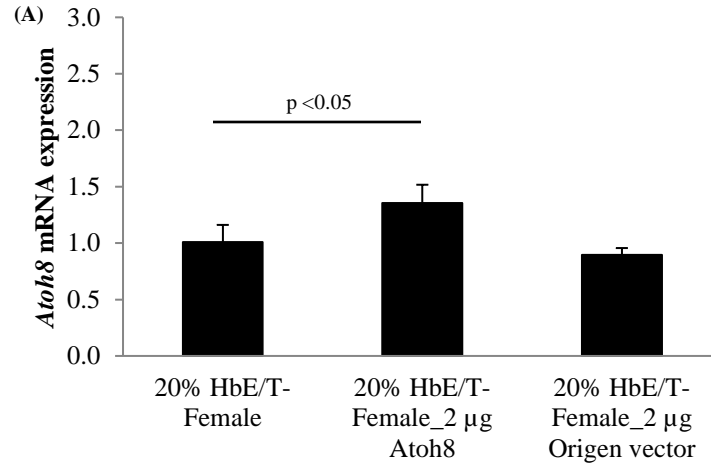
5 Promoter activity was expressed as the relative luciferase activity to the untransfected ATOH8-

6 FLAG group. The samples were determined in triplicate. Values are expressed as mean±SD for

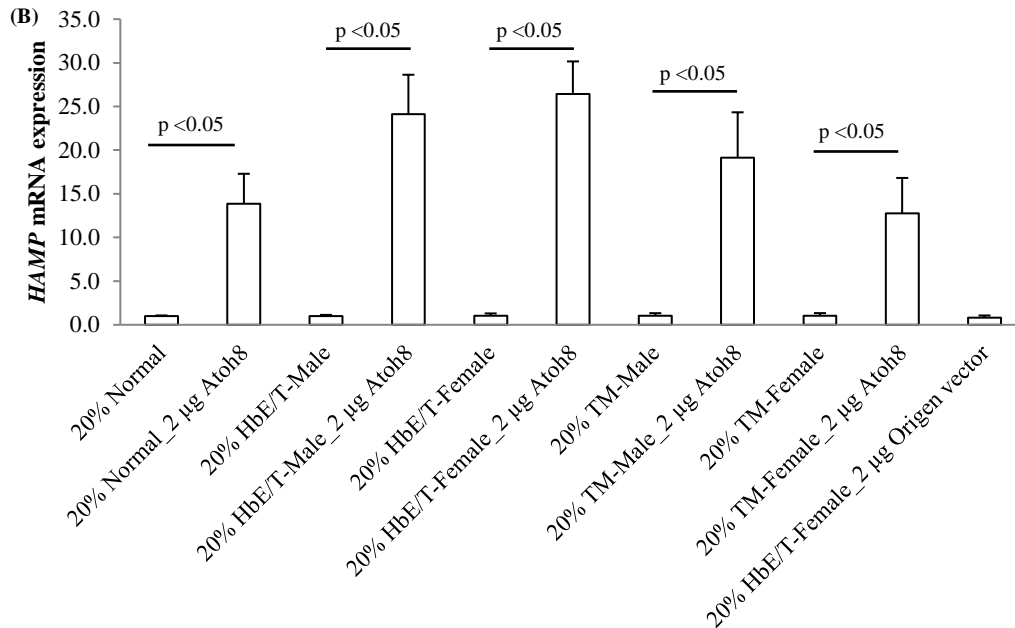
7 the fold change as compared to the untransfected ATOH8-FLAG group. *p<0.05 when compared

8 with the untransfected ATOH8-FLAG group.

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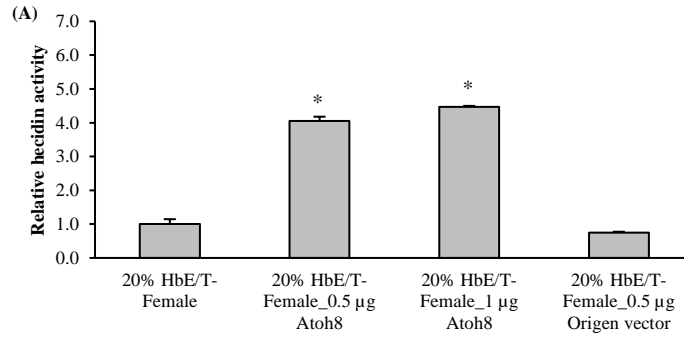


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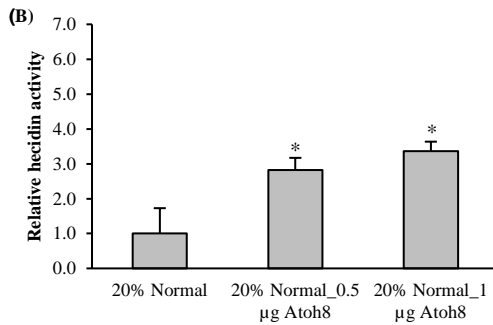


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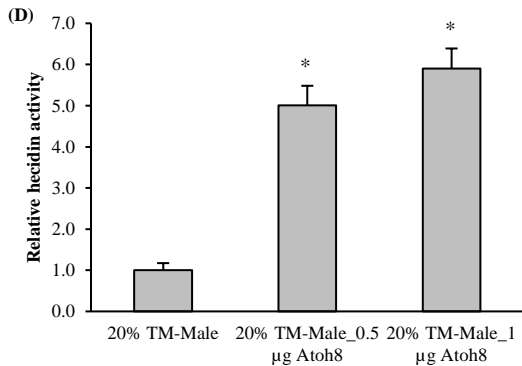
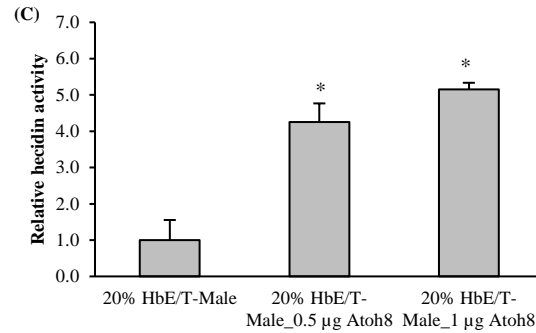
3 **Figure 6** *Atoh8* and *HAMP* mRNA expression in HEK293 cells transfected with (2 μg)
 4 ATOH8-FLAG expression plasmid or empty plasmid (Origen vector) and grown in DMEM
 5 medium supplemented with 20% human sera for 24 hours. Relative *Atoh8* (A) and *HAMP* (B)
 6 mRNA expression from qPCR analysis were acquired by normalizing *Atoh8* mRNA or *HAMP*
 7 mRNA to *RPL19* mRNA. The samples were determined in triplicate. Values are expressed as
 8 mean±SD for the fold change as compared to the untransfected ATOH8-FLAG group.



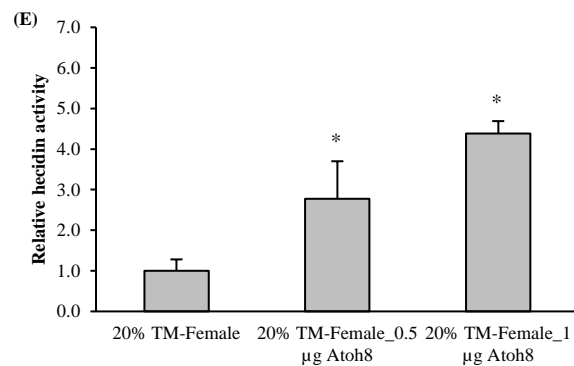
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4 **Figure 7 *HAMP* promoter activity in HEK293 cells co-transfected with *HAMP* reporter**
 5 **plasmid, TK-renilla and (0.5 and 1 µg) ATOH8-FLAG expression plasmid (or Origen**
 6 **vector) and grown in DMEM medium supplemented with 20% human sera (A: Female**
 7 **HbE/β-thalassemia, B: Normal, C: Male HbE/β-thalassemia, D: Male β-thalassemia major,**
 8 **D: Female β-thalassemia major) for 24 hours. Promoter activity was expressed as the relative**
 9 **luciferase activity to the untransfected ATOH8-FLAG group. The samples were determined in**
 10 **triplicate. Values are expressed as mean±SD for the fold change as compared to the untransfected**
 11 **ATOH8-FLAG group. *p<0.05 when compared with the untransfected ATOH8-FLAG group.**