Iron homeostasis in a mouse model of thalassemia intermedia is altered between adolescence and adulthood

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Background. Iron overload is one of common complications of β-thalassemia. Systemic iron homeostasis is regulated by iron- regulatory hormone, hepcidin, which inhibits intestinal iron absorption and iron recycling by reticuloendothelial system. In addition, body iron status and requirement can be altered with age. In adolescence, iron requirement is increased due to blood volume expansion and growth spurt. Heterozygous β-globin knockout mice ($Hbb^{th3/+}$; BKO) is a mouse model of thalassemia widely used to study iron homeostasis under this pathological condition. However, effects of age on iron homeostasis, particularly the expression of genes involved in hemoglobin metabolism as well as erythroid regulators in the spleen, during adolescence have not been explored in this mouse model. **Methods.** Iron parameters as well as the mRNA expression of hepcidin and genes involved in iron transport and metabolism in wildtype (WT) and BKO mice during adolescence (6-7 weeks old) and adulthood (16-20 weeks old) were analyzed and compared by 2-way ANOVA. **Results.** The transition of adolescence to adulthood was associated with reductions in duodenal iron transporter mRNA expression and serum iron levels of both WT and BKO mice. Erythrocyte parameters in BKO mice remained abnormal in both age groups despite persistent induction of genes involved in hemoglobin metabolism in the spleen and progressively increased extramedullary erythropiesis. In BKO mice, adulthood was associated with increased liver hepcidin and ferroportin mRNA expression along with splenic erythroferrone mRNA suppression compared to adolescence. **Conclusion.** Our results demonstrated that iron homeostasis in a mouse model of thalassemia intermedia was altered between adolescence and adulthood. The present study underscores the importance of the age of thalassemic mice in the study of molecular or pathophysiological changes under thalassemic condition.

1 **Iron homeostasis in a mouse model of thalassemia**

2 **intermedia is altered between adolescence and**

3 **adulthood**

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Abstract

Background. Iron overload is one of common complications of β-thalassemia. Systemic iron homeostasis is regulated by iron- regulatory hormone, hepcidin, which inhibits intestinal iron absorption and iron recycling by reticuloendothelial system. In addition, body iron status and requirement can be altered with age. In adolescence, iron requirement is increased due to blood volume expansion and growth spurt. Heterozygous β-globin knockout mice ($Hbb^{th3/+}$; BKO) is a mouse model of thalassemia widely used to study iron homeostasis under this pathological condition. However, effects of age on iron homeostasis, particularly the expression of genes involved in hemoglobin metabolism as well as erythroid regulators in the spleen, during adolescence have not been explored in this mouse model.

Methods. Iron parameters as well as the mRNA expression of hepcidin and genes involved in iron transport and metabolism in wildtype (WT) and BKO mice during adolescence (6-7 weeks old) and adulthood (16-20 weeks old) were analyzed and compared by 2-way ANOVA.

Results. The transition of adolescence to adulthood was associated with reductions in duodenal iron transporter mRNA expression and serum iron levels of both WT and BKO mice. Erythrocyte parameters in BKO mice remained abnormal in both age groups despite persistent induction of genes involved in hemoglobin metabolism in the spleen and progressively increased extramedullary erythropiesis. In BKO mice, adulthood was associated with increased liver hepcidin and ferroportin mRNA expression along with splenic erythroferrone mRNA suppression compared to adolescence.

Conclusion. Our results demonstrated that iron homeostasis in a mouse model of thalassemia intermedia was altered between adolescence and adulthood. The present study underscores the importance of the age of thalassemic mice in the study of molecular or pathophysiological changes under thalassemic condition.

Keywords Heterozygous β-globin knockout mice, hepcidin, age, thalassemia, iron transporters

Introduction

50

51   Iron, one of essential trace elements, is involved in several biological processes such as

52 oxidative phosphorylation and hemoglobin synthesis (*Jandl et al., 1959*; *Pollycove & Mortimer,*

53 *1961*; *Hentze et al., 2004*). Iron is transported in the plasma by apotransferrin protein.

54 Transferrin-bound iron is taken up into cells by transferrin receptor 1 (TfR1) for cellular storage

55 or utilization. In erythroid cells, the acquired iron is used for the production of hemoglobin

56 which involves several proteins including mitoferrin 1 (Mfrn1), a mitochondrial iron transporter,

57 and 5-aminolevulinic acid synthase 2 (ALAS2), an enzyme catalyzing the first reaction of

58 erythroid heme biosynthetic pathway (*Shaw et al., 2006*; *Paradkar et al., 2009*; *Amigo et al.,*

59 *2011*; *Lane et al., 2015*). Reticuloendothelial (RE) cells are responsible for the reutilization of

60 iron in hemoglobin of senescent red blood cells. Upon erythrophagocytosis of senescent

61 erythrocytes by RE cells, hemoglobin is degraded by heme oxygenase-1 (HO-1) and iron is

62 released into the circulation for reutilization. This RE iron recycling process accounts for the

63 majority of iron in the plasma. The minority of plasma iron is acquired from intestinal iron

64 absorption (*Kong et al., 2014*; *Wallace, 2016*).

65   Several molecules are involved in the absorption of iron. Firstly, dietary iron (Fe^{3+}) is

66 reduced by duodenal cytochrome b (Dcytb) located at the apical membrane of enterocytes

67 (*McKie et al., 2001*). The resultant ferrous is taken up into enterocytes by divalent metal

68 transporter 1 (DMT1) (*Gunshin et al., 1997*) and then transferred into the circulation by an iron

69 efflux protein, ferroportin (*McKie et al., 2000*; *Donavan et al., 2000*; *Abboud & Haile, 2000*).

70 The ferrous is subsequently oxidized by hephaestin into ferric which is then transported along the

71 plasma by apotransferrin (*Vulpe et al., 1999*). Ferroportin not only involves in cellular export of

72 dietary iron but also facilitates iron efflux from splenic macrophages and hepatocytes. In

73 addition to intestinal iron uptake, DMT1 is also involved in the transport of transferrin-bound

74 iron from endosome into cytoplasm.

75   Cellular iron homeostasis is regulated by iron responsive elements (IRE), which are

76 located at either 5' or 3' untranslated region of several mRNAs encoding proteins related to

77 cellular iron metabolism including TfR1, DMT1, ferroportin and ALAS2 (*Koeller et al., 1989*;

78 *Cox et al., 1991*; *Dandekar et al., 1991*; *Abboud & Haile, 2000*; *McKie et al., 2000*; *Hubert &*

79 *Hentze, 2002*). The expression of these proteins is post-transcriptionally controlled in response

80 to cellular iron levels through the interaction between IRE and iron regulatory protein (IRP).

81   TfR1 and DMT1 expression is increased under iron depletion state and vice versa under iron

82   repletion. On the other hand, the expression of ferroportin and ALAS2 is suppressed in response

83   to iron deficiency and enhanced during iron loading.

84   Systemic iron homeostasis is regulated by hepcidin, the liver-secreted iron regulatory

85   hormone, which binds ferroportin and induces its internalization and degradation (*Nemeth et al.,*

86   *2004*). Consequently, intestinal iron absorption and RE iron recycling is inhibited resulting in

87   reduced serum iron levels. The expression of hepcidin is influenced by several factors. Body

88   iron status and inflammation have been shown induce hepcidin expression whereas

89   erythropoietic iron demand and hypoxia are hepcidin suppressors (*Pigeon et al., 2001*; *Nicolas et*

90   *al., 2002a*; *Nicolas et al., 2002b*). Bone morphogenetic protein 6 (Bmp6) has been shown to

91   play a crucial role in hepcidin induction in response to iron stores (*Andriopoulos et al., 2009*). In

92   addition to the aforementioned factors, ineffective erythropoiesis can also suppress the

93   expression of hepcidin. Growth differentiation factor 15 (Gdf15), twisted gastrulation 1 (Twsg1)

94   and erythroferrone (Erfe) which are produced by erythroid precursors have been proposed to be

95   potential candidates for hepcidin regulator under such conditions including thalassemia (*Tanno*

96   *et al., 2007*; *Tanno et al., 2009*; *Kautz et al., 2014*).

97   Thalassemia is a hematological disease caused by mutations of globin-encoding genes or

98   their promoters leading to decreased production of the respective globin chains. In β-

99   thalassemia, the production of β-globin is reduced or absent leading to the accumulation of

100   unmatched α-globin, erythroid cell death, ineffective erythropoiesis and, subsequently, anemia.

101   Furthermore, systemic iron overload occurs as a result of increased intestinal iron absorption

102   and/or regular blood transfusion with inadequate iron chelation (*Weatherall & Clegg, 2001*;

103   *Higgs et al., 2012*). Animal models especially mouse (*Mus Musculus*) is a model that most

104   widely used to study the pathophysiological changes or treatment of human disease (*Capecchi,*

105   *2005*). Heterozygous β-globin knockout mice (Hbb$^{th3/+}$; BKO), one of commonly utilized

106   thalassemia mouse model, were generated by heterozygous deletion of both murine β-globin

107   genes (*Hbb-b1* and *Hbb-b2*) (*Detloff et al., 1994*; *Yang et al., 1995*). Phenotypic characterization

108   of this mouse model includes mild-to-moderate anemia, growth retardation, ineffective

109   erythropoiesis, extramedullary erythropoiesis and parenchymal iron loading in several tissues

110   such as liver, spleen and heart which resemble clinical features of thalassemia in human (*Yang et*

111   *al., 1995*).

112   Several factors including age, gender and strain have been shown to influence iron

113   parameters as well as the expression of hepcidin and other iron regulatory genes (*Ahluwalia et*

114   *al.,2000*; *Courselaud et al., 2004*; *Krijt et al., 2004*; *Weizer-Stern et al., 2006*; *Hahn et al., 2009*;

115   *McLachlan et al., 2017*). Previous study reported that iron levels in various tissues were

116   generally higher in aged mice than younger adult mice (*Hahn et al., 2009*). The expression of

117   genes involved in iron metabolism was proposed to be dynamic as a result of age and iron stores

118   (*De Franceschi et al., 2006*). Additionally, previous study proposed that age must be considered

119   in the studies of iron metabolism (*Chen et al., 2009*). It is noteworthy that mice at different ages

120   might have distinctive responses to alterations in iron metabolism. Indeed, the study in β-

121   thalassemia mouse model ($Hbb^{th3}/^{+}$) aged 2, 5 and 12 months demonstrated different systemic

122   iron regulatory responses in young adult and elderly thalassemic mice (*Gardenghi et al., 2007*).

123   Although BKO mice have been widely used as an animal model in thalassemia research, little is

124   known whether the expression of hepcidin along with major iron transporters in these mice differ

125   across different age groups particularly in adolescence. Murine adolescent age is defined as the

126   postnatal period ranging from weaning (PND 21) to adulthood (PND 60) (*Laviola et al., 2003*).

127   It is also suggested that adult mice should be at least 3 months of age as most biological

128   processes and structures continue to rapidly grow or mature after the age of sexual maturation

129   (35 days) until roughly 3 months old (*Flurkey et al., 2007*). Moreover, iron status and iron

130   homeostasis might be altered in adolescence as a result of increased body iron requirement for

131   growth and development (*Beard, 2000*). Thus, the present study aims to explore the effects of

132   age on iron parameters as well as the mRNA expression of hepcidin and major iron transport

133   machineries in WT and thalassemic mouse model aged 6-7 weeks old and 16-20 weeks old,

134   which represent adolescence and adulthood, respectively.

135

Materials & Methods

137   **Animal**

138   Male C57BL/6 wild type (WT) and heterozygous β-globin knockout mice ($Hbb^{th3/+}$;

139   BKO) at two age groups: 6-7 weeks old and 16-20 weeks old were used (5 mice per each of the

140   age group). All mice were obtained from the Thalassemia Research Center, Institute of

141   Molecular Biosciences, Mahidol University, Thailand. The mice were given rodent chow (C.P.

142   mice feed 082G containing 180 ppm of iron, Perfect Companion Group, Thailand) and water *ad*

143 *libitum*. The temperature and humidity were maintained at $25 \pm 2°C$ and $55 \pm 10\%$, respectively,

144 with 12 hours light/dark cycle. The mice were sacrificed and blood samples were collected by

145 cardiac puncture. Liver, spleen and duodenum samples were snapped frozen and stored at -80℃.

146 Animal protocols were approved by Institute of Molecular Biosciences Animal Care and Use

147 Committee, Mahidol University, Thailand (COA.NO.MB-ACUC 2016/003).

148

149 **Measurement of hematological and iron parameters**

150 Hematological parameters were analyzed using an automated hematological analyzer

151 (Mindray, China). Serum iron level was determined using QuantiChrome iron assay kit

152 (BioAssay System, USA) according to the manufacturer's protocol. Liver and spleen non-heme

153 iron contents were determined by a modification of the method of Foy et al. (*Foy et al.,1967*) as

154 described by Simpson and Peters (*Simpson & Peters, 1990*).

155

156 **Histopathological studies**

157 Liver, spleen and duodenal tissue samples were fixed in 10% formalin buffer. The fixed

158 tissue samples were then dehydrated, embedded in paraffin, and sectioned at 4-μm thickness.

159 Tissue sections were stained with hematoxylin and eosin (H&E) for morphological examination

160 and Perl's Prussian blue for iron accumulation according to standard protocols. The stained

161 slides were analyzed using a Nikon ECLIPSE 80i light microscope (Nikon, Japan).

162 The amounts of mononuclear, polymorphonuclear and hematopoietic cells in the liver

163 were evaluated in 10 non-overlapping areas acquired by random sampling. The numbers of cells

164 were scored using a grading criteria described by Yatmark et al. (*Yatmark et al., 2014*) as

165 follows: score 0 = absent ; score 1 = mild (1 - 10 cells) ; score 2 = moderate (11 - 50 cells) ;

166 score 3 = severe (more than 50 cells).

167 For iron accumulation assessment, the whole tissue section of each mouse (5 mice per

168 group) was examined and the overall extent of iron deposition in each mouse was estimated by a

169 modification of grading system described by Barton et al. (*Barton et al.,1995*) and Yatmark et al.

170 (*Yatmark et al., 2014*) as follows:

171 a) Liver iron deposition: score 0 = no visible iron deposition; score 1 = slight iron

172 deposition in the cytoplasm of Kupffer cells ; score 2 = prominent iron accumulation in Kupffer

173 cells ; score 3 = iron deposition in hepatocytes ; score 4 = iron deposition in hepatocytes and

174 fibrous tissue of portal tracts or septa

175   b) Splenic iron deposition: score 0 = no visible iron deposition ; score 1 = indicated iron

176 deposit in marginal sinus of spleen; score 2 = densely aggregated iron deposition ; score 3,=

177 clumps of iron accumulation : score 4 = clumps of iron aggregation with frequent iron clumps.

178   c) Duodenal iron deposition: score 0 = no visible iron deposition; score 1 = iron deposits

179 in the supranuclear region of enterocyte; score 2 = densely aggregated iron deposition ; score 3,=

180 visible clumps of iron accumulation ; 4 = clumps of iron aggregation with frequent iron clumps

181

182 **Quantitative RT-PCR**

183   RNA was extracted from the liver, spleen and duodenum using TRIzol reagent (Ambion,

184 USA), and complementary DNA was synthesized using a Tetro cDNA synthesis kit (Bioline,

185 USA) as per manufacturers' protocols. Quantitative RT-PCR was performed using the CFX96

186 Thermal Cycler (Bio-Rad, USA). The gene expression was normalized to β-actin (*Actb*)

187 expression. The results were presented as minus delta Ct values (Ct $_{target}$ − Ct $_{Actb}$) (*Livak &*

188 *Schmittgen, 2001*). The sequence of gene-specific primers is listed in Table 1.

189

190 **Statistical analysis**

191   All data were expressed as mean ± standard error of the means (SEM). The comparison

192 of the means across the two age groups was performed by 2-way ANOVA with Bonferroni post-

193 hoc test. A *P*-value less than 0.05 was considered significant. All statistical analyses were

194 performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

195

196

197 # Results

198   **Iron parameters, but not hematological parameters, of thalassemic mice were**

199 **affected by age**

200   To explore whether hematological parameters were affected by the age of mice, EDTA

201 blood samples from WT and BKO mice aged 6-7 weeks and 16-20 weeks were analyzed for

202 complete blood count. As shown in Table 2, significant effects of phenotype on several

203 hematological parameters were observed. BKO mice had lower hemoglobin, hematocrit, MCV

204 and RBC count along with higher RDW than age-matched WT which corresponded with

205 thalassemia phenotype. Furthermore, such findings remained unaltered between adolescent and

206 adult BKO mice. In WT mice, adulthood was associated with increased RBC count,

207 hemoglobin, hematocrit along with reduced MCV and RDW compared to adolescence.

208   Serum iron levels as well as non-heme iron levels in the liver and spleen were measured

209 to determine body iron status. Increased liver and spleen non-heme iron levels were observed in

210 BKO mice whereas serum iron levels did not differ from WT mice (Table 2). In both WT and

211 BKO mice, a significant reduction in serum iron levels were observed in adult mice compared to

212 adolescent mice while liver non-heme iron levels were unaltered. Interestingly, adulthood was

213 associated with a significant increase in spleen non-heme iron levels only in BKO mice.

214   Perl's Prussian blue staining revealed iron accumulation in the liver and spleen of BKO

215 mice (Fig. 1C-D, 1G-H). Notably, iron deposition was mainly confined in hepatic Kupffer cells

216 and splenic macrophages in both red and white pulps. Additionally, a weakly positive iron

217 staining was observed in the supranuclear region of duodenal enterocytes (Fig. 1K-L). The

218 degree of iron staining was relatively higher in the spleen than the liver and duodenum (Table 3).

219 In agreement with non-heme iron results, an increase in iron deposition score was found in the

220 spleen of adult BKO group compared to adolescent BKO mice whereas liver and duodenal iron

221 deposition score was unaltered between different age groups.

222

223 **The extent of extramedullary hematopoiesis in thalassemic mice was progressively**

224 **increased with increasing age**

225   Microscopic examination of liver and spleen tissue samples revealed hematopoietic and

226 mononuclear cell infiltration in the liver and spleen of BKO mice which was suggestive for the

227 presence of extramedullary hematopoiesis (Fig. 2C-D, 2G-H). As shown in Table 4, the livers of

228 adult BKO mice had increased number of mononuclear cell infiltration and hematopoietic cells

229 than adolescent BKO mice suggesting that the extent of extramedullary hematopoiesis

230 progressively increased at least during the studied age range.

231   Furthermore, genes involved in hemoglobin synthesis (*Tfrc*, *Slc25a37* and *Alas2*) and

232 degradation (*Hmox1*) were induced in the spleen of BKO mice compared to WT mice (Fig. 3).

233 Interestingly, mRNA levels of these genes were significantly decreased in adult WT mice

234 compared to adolescent counterpart while the expression in BKO mice was similar between the

235 two age groups.

236

Thalassemic mice had decreased liver hepcidin mRNA expression relative to liver non-heme iron contents during both adolescence and adulthood

239   Quantitative RT-PCR revealed that the levels of liver hepcidin mRNA were similar
240 between WT and BKO mice during adolescence (Fig. 4A). In adulthood, hepcidin mRNA
241 expression in BKO mice was higher than adolescent mice. On the contrary, hepcidin mRNA
242 levels in WT were not affected by age. To account for liver iron overload, the mRNA expression
243 of hepcidin was corrected by liver non-heme iron contents. As shown in Fig. 4B, BKO mice in
244 both age groups had lower hepcidin mRNA levels relative to liver non-heme iron contents than
245 WT mice. No effect of age on this parameter was observed.

246   The mRNA expression of upstream hepcidin regulators in the liver and spleen was also
247 determined. Liver Bmp6 mRNA levels as well as splenic mRNA expression of Gdf15, Twsg1
248 and Erfe were higher in BKO mice compared to WT mice (Fig. 4 C-F). In general, the mRNA
249 levels of these hepcidin regulators was not altered between adolescent and adult mice except
250 splenic Erfe mRNA expression which was downregulated in adult BKO mice.

251

Iron transporter mRNA expression was differentially affected by thalassemia and age

253   In the liver, DMT1 mRNA expression was not affected by thalassemia and age (Fig. 5A).
254 In contrast, ferroportin mRNA levels were increased in BKO mice (Fig. 5B). A significant
255 ferroportin mRNA induction was observed in adult BKO mice compared to adolescent
256 counterpart.

257   In addition, increased splenic mRNA levels of DMT1 and ferroportin were noted in BKO
258 mice but the expression was not altered between adolescent and adult mice (Fig. 5 C-D).

259   As shown in Fig. 6, thalassemia had significant positive effects of the mRNA expression
260 of Dytb and DMT1 in the duodenum. Furthermore, Dcytb and DMT1 mRNA levels were
261 significantly reduced in adult WT and BKO mice compared to respective adolescent group. On
262 the other hand, duodenal ferroportin mRNA expression was not significantly affected by
263 phenotype or age.

264

Discussion

266

267   Our study reveals that iron parameters and the expression of genes involved in iron

268   homeostasis of WT and thalassemic mice differ between adolescence and adulthood. Although,

269   previous study has determined hematological parameters and the expression of iron genes in

270   thalassemic mice at different ages, the study focused on gene expression in the liver (*De*

271   *Franceschi et al., 2006*). In addition, it was conducted in only in mature adult and aged mice.

272   The study by Gardenghi et al. utilized thalassemic mice aged 2, 5 and 12 months to compare iron

273   status and iron homeostasis, however, the expression of splenic iron transporters as well as genes

274   involved in hemoglobin synthesis and genes encoding erythriod regulators (Erfe, Gdf15, Twsg1)

275   was not explored (Gardenghi et al., 2007).

276   In accordance with previous studies (*Jamsai et al., 2005*; *Nai et al., 2012*; *Upanan et al.,*

277   *2015*), we observed thalassemia intermedia phenotype in BKO mice during adolescence and

278   adulthood as characterized by microcytic anemia, extramedullary hematopoiesis as well as iron

279   accumulation in the liver and spleen. In the present study, hematological results revealed

280   relatively lower red blood cell count and hemoglobin levels in both WT and BKO mice than

281   previously reported values (*De Franceshi et al., 2006*; *Weizer-Stern et al., 2006*; *Gardenghi et*

282   *al., 2007*; *Vogiatzi et al., 2010*; *Nai et al., 2012*; *Gelderman et al., 2015*; *Kautz et al., 2015*; *Li et*

283   *al., 2017*). However, comparable hematological parameters were also reported in 20-week old

284   WT and BKO mice obtained from the same animal facility as our study (*Wannasuphaphol et al.,*

285   *2005*). Furthermore, we found that adolescent WT mice had significantly lower RBC count,

286   hemoglobin and hematocrit along with significantly higher MCV and RDW than adult WT mice.

287   Physiological changes in hematological parameters of young mice including lower hemoglobin,

288   lower hematocrit, higher MCV has previously been documented (*Everds, 2007*). In agreement

289   with our results on RDW values, variable red blood cells morphology has also been noted in

290   young mice (*Bannerman, 1983*). In contrast to WT mice, the hematological abnormalities in

291   BKO mice persisted in both age groups indicating for the persistence of thalassemic phenotype

292   in these mice. In addition to age-associated hematological changes, increased serum iron levels

293   during adolescence were observed in both WT and BKO mice. A similar trend has previously

294   been reported in normal mice and rats (*Lesbordes-Brion et al., 2006*; *Kong et al., 2015*). We

295   speculate that serum iron levels were increased during adolescence as a result of increased iron

296   requirement for erythropoiesis.

297   Several proteins are involved in the synthesis of hemoglobin in red blood cells. TfR1 is
298   the essential iron acquisition machinery of maturing erythroid cells (*Kong et al., 2014*;
299   *Gammella et al., 2017*), while Mfrn1 and ALAS2 are required for heme biosynthesis (*Hentze et*
300   *al., 2004*; *Amigo et al., 2011*; *Chiabrando et al., 2014*). The expression of Mfrn1 and ALAS2 is
301   transcriptionally regulated by transcription factor GATA-1 during erythroid maturation (*Amigo*
302   *et al., 2011*; *Tanimura et al., 2016*). In the present study, quantitative real-time PCR reveals that
303   genes involved in heme biosynthesis and degradation were induced the spleen of in BKO mice.
304   Interestingly, adulthood was associated with reduced splenic mRNA expression of these genes in
305   WT mice whereas the mRNA levels in adult BKO mice were persistently increased. In
306   accordance, mRNA levels of DMT1, which plays central role in erythroid iron transport across
307   endosomes (*Canonne-Hergaux et al., 2001*), were also induced in adolescent and adult BKO
308   mice. These findings indicate that extramedullary hematopoiesis in BKO mice was induced
309   during both age groups. In accordance, histopathological examination also showed that the
310   extent of extramedullary hematopoiesis in BKO mice was progressively increased with
311   increasing age. Furthermore, the presence of anemia despite enhanced extramedullary
312   erythropoiesis in BKO mice is indicative for ineffective erythropoiesis.
313   In this study, both tissue non-heme iron measurement and histopathological examination
314   revealed progressive iron accumulation in the spleen of thalassemic mice particularly in
315   adulthood. In contrast, liver iron deposition was not significantly altered between adolescent and
316   adult BKO mice. Notably, Gardenghi et al. (*Gardenghi et al., 2007*) reported a differential pace
317   of iron accumulation in BKO mice between the spleen and hepatic Kupffer cells. In accordance
318   with our findings, the previous work by Kautz et al. (*Kautz et al., 2015*) demonstrated that iron
319   content in the liver of BKO mice was markedly increased during early adolescence and reached
320   plateau during late adolescence and early adulthood. The alteration in the pattern of tissue iron
321   deposition between adolescence and adulthood suggests that systemic iron homeostasis could
322   differ with age.
323   The expression of hepcidin, a systemic iron regulatory peptide, is regulated by several
324   factors. Under thalassemic conditions, hepcidin might be concurrently affected by systemic iron
325   loading, anemia and ineffective erythropoiesis. It has been proposed that although
326   erythropoiesis, iron status, and inflammation all contribute to variation in hepcidin expression,
327   erythropoietic drive appears to be the strongest contributor (*Karafin et al., 2015*). In agreement

328 with the work by Gardenghi et al. (*Gardenghi et al., 2007*), our data suggests that hepcidin

329 expression is determined by both anemia and iron overload which have opposing effects. The

330 net effect of these two factors on hepcidin expression, which may vary depending on age. In this

331 study, the expression of Bmp6, a positive hepcidin regulator, and erythroid-secreted hepcidin

332 suppressors (Erfe, Gdf15 and Twsg1) was induced in thalassemic mice. The lower hepcidin

333 mRNA levels relative to liver non-heme iron contents in BKO mice suggest that hepcidin was

334 influenced by erythroid regulators under thalassemic condition. On the contrary, liver hepcidin

335 mRNA levels which reflect the net effects of hepcidin regulators did not differ between WT and

336 BKO mice during adolescence. Therefore, we speculate that Bmp6 and erythroid regulators both

337 contributed on hepcidin expression in adolescent thalassemic mice. In accordance with previous

338 studies, we observed increased hepcidin expression in thalassemic mice during adulthood (*Nai et*

339 *al., 2012*; *Kumfu et al., 2016*) compared to adolescence. However, the hepcidin-to-liver iron

340 ratio in BKO mice did not alter between the two age groups suggesting that the increase in

341 hepcidin mRNA levels was appropriate for the increase in liver iron contents. Additionally, we

342 observed decreased splenic Erfe mRNA expression in adult BKO mice compared to adolescent

343 counterpart. Interestingly, Erfe has recently been demonstrated to inhibit BMP6-induced

344 hepcidin expression (*Arezes et al., 2018*). Accordingly, the relative reduction in Erfe mRNA

345 expression in thalassemic mice during adulthood would allow hepcidin induction by BMP6 in

346 response to increased liver iron accumulation. Notably, the previous study demonstrated that

347 Erfe mRNA expression was consistently induced in the spleen of thalassemic mice during the

348 age of 3 - 12 weeks (*Kautz et al., 2015*). A study to further explore whether Erfe mRNA levels

349 in the spleen is altered in adult and aged thalassemic mice should be conducted.

350   We demonstrated that ferroportin mRNA expression in the spleen was induced in BKO

351 mice and corresponded with the induction of genes involved in hemoglobin synthesis and

352 degradation. Therefore, it is possible that increased erythropoiesis and subsequent degradation

353 of erythroid cells are responsible for the increased splenic ferroportin expression in adult BKO

354 mice. In agreement, erythrophagocytosis has been shown to induce ferroportin and HO-1

355 transcription in vitro (*Knutson et al*., 2003; *Delaby et al*., 2008). Furthermore, the expression of

356 ferroportin in iron-recycling macrophages is upregulated by both heme and iron acquired from

357 the degradation of hemoglobin in senescent red blood cells. Heme has been shown to

358 transcriptionally induce ferroportin whereas the regulation of ferroportin expression by iron

359   occurs at the translation level though IRP-IRE interaction (*Marro et al., 2010*; *Drakesmith et al.,*

360   *2015*). In the liver, ferroportin mRNA expression was induced in adolescence and adult

361   thalassemic mice, possibly, as a result of liver iron accumulation, which were present in both

362   periods of lifespan. In line with our findings, iron dextran administration has been shown to

363   induce liver ferroportin mRNA expression in C3HeB/FeJ mice (*Liu et al., 2005*). In contrast to

364   ferroportin, we found that DMT1 mRNA expression in the liver was not affected by thalassemia

365   or age. In thalassemia, the increase in iron levels in the circulation exceeds the binding capacity

366   of apotransferrin resulting in the presence of non-transferrin bound iron (NTBI). Notably,

367   previous study reported that DMT1 does not play an essential role in the uptake of NTBI in the

368   liver (*Wang & Knutson, 2013*). Further studies should be performed to delineate the roles of

369   other NTBI transporters such as Zip14 in the pathophysiology of liver iron overload under

370   thalassemic condition and to determine whether the expression of these transporters differs with

371   age.

372   Intestinal iron hyperabsorption as a result of hepcidin downregulation has been

373   previously reported in thalassemic mice (*Gardenghi et al., 2007*). In agreement, we

374   demonstrated significant effects of thalassemia on the mRNA expression of apical iron transport

375   machineries, Dcytb and DMT1, in the duodenum. Interestingly, reduced Dcytb and DMT1

376   mRNA expression was observed in the duodenum of adult WT and BKO mice compared to

377   adolescent counterparts. These changes in duodenal iron transporter expression also coincided

378   with the reduction in serum iron levels of both WT and BKO mice during adulthood. It has

379   previously been shown that hepcidin expression inversely correlates with duodenal iron

380   transporters mRNA expression and iron absorption in rats (*Frazer et al., 2002*; *Frazer et al.,*

381   *2004*; *Millard et al., 2004)*. However, our study observed Dcytb and DMT1 downregulation in

382   adult WT mice compared to adolescent WT mice although hepcidin expression was unaltered

383   between these two age groups. Therefore, we speculate that hepcidin did not directly involve in

384   the altered Dcytb and DMT1 expression between adolescence and adulthood. Previous study in

385   rat reported that the expression of Dcytb, DMT1 and ferroportin was significantly affected by

386   age (*Kong et al., 2015*). Moreover, hypoxia-inducible factor 2 alpha (HIF-2α) has been shown

387   to transcriptionally induce Dcytb and DMT1 in response to iron deficiency (*Mastrogiannaki et*

388   *al., 2009*; *Shah et al., 2009*). In the present study, we not only found anemia in BKO mice but

389   also reduced levels of RBC count, hemoglobin and hematocrit in adolescent WT mice compared

390 adult WT mice. It is therefore possible that anemia-associated hypoxia was responsible for the

391 upregulation of intestinal iron transporters observed in our adolescent mice. The mechanisms of

392 age-associated changes in duodenal iron transporters including the possible roles of HIF-2α

393 remain to be elucidated.

394   In summary, our study demonstrated that several hematological and iron homeostatic

395 parameters were different between adolescence and adulthood. As the mice progressed from

396 adolescence to adulthood, duodenal iron transporter mRNA expression and serum iron levels of

397 both WT and BKO mice were decreased. Reduced splenic expression of genes involved in

398 hemoglobin metabolism as well as alteration in erythrocyte parameters were found only in WT

399 mice. In BKO mice, erythrocyte abnormalities along with induction of genes involved in

400 hemoglobin metabolism in the spleen were persistent in both adolescence and adulthood.

401 Interestingly, adulthood was associated with increased liver hepcidin and ferroportin mRNA

402 expression along with splenic Erfe mRNA suppression only in BKO mice. The limitations of the

403 present study include the lack of findings on serum hepcidin and Erfe levels as well as gene

404 expression in the bone marrow. In addition, the expression of iron transporters at the protein

405 level should be performed in future studies.

406

407 **Conclusions**

408   Our study demonstrated that iron homeostasis in a mouse model of thalassemia

409 intermedia differed between adolescence and adulthood. During adolescence, increased mRNA

410 expression of duodenal iron transporters was associated with liver iron accumulation. In adult

411 BKO mice, the induction of splenic Erfe and duodenal iron transporters was diminished

412 compared to adolescent BKO mice. Furthermore, extramedullary erythropoiesis is enhanced and

413 the spleen becomes preferential site of tissue iron loading. The present study demonstrates that

414 iron homeostasis in thalassemia intermedia might be altered with age *per se* and disease

415 progression. The present study underscores the significance of age on the expression of genes

416 involved in iron metabolism as well as the pathophysiology of iron loading in thalassemia

417 intermedia. Therefore, the age of thalassemic mice should be considered in the study of iron

418 homeostasis under thalassemic condition

419

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423

424

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Competing Interests

The authors declare that they have no competing interests.

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687 **Figures legends**
688 **Figure 1 Perl's Prussian blue staining of tissue samples from WT and BKO mice at**
689 **adolescence and adulthood.** Perl's Prussian blue staining was performed in representative
690 paraffin-embedded tissue sections from the liver (A-D), spleen (E-H) and duodenum (I-L) of
691 male WT and BKO mice aged 6-7 weeks and 16-20 weeks. The blue stain represents iron
692 accumulation. The arrows indicate iron accumulation in the supranuclear region of enterocytes.
693 (Nikon ECLIPSE 80i light microscope ; magnification x400)

694

695   **Figure 2 Hematoxylin and eosin staining of tissue samples from WT and BKO mice at**

696   **adolescence and adulthood.**   Hematoxylin and eosin staining of representative paraffin-

697   embedded tissue sections from the liver (A-D), spleen (E-H) and duodenum (I-L) of male WT

698   and BKO mice aged 6-7 weeks and 16-20 weeks. The arrows indicate mononuclear cells and

699   hematopoietic cells in the liver and spleen. (Nikon ECLIPSE 80i light microscope ;

700   magnification x400)

701

702   **Figure 3 The expression of genes involved in hemoglobin biosynthesis and degradation in**

703   **the spleen of WT and BKO mice at adolescence and adulthood.** Quantitative RT-PCR of

704   TfR1 (A), Mfrn1 (B), ALAS2 (C) and HO-1 (D) mRNA from the spleen of WT and BKO mice

705   aged 6-7 weeks and 16-20 weeks. Values are presented as means and SEM for minus delta Ct

706   values (n = 5 per group). Statistical analysis was performed by 2-way ANOVA with Bonferroni

707   post-hoc test.

708

709   **Figure 4 The expression of hepcidin and upstream regulators of hepcidin in WT and BKO**

710   **mice at adolescence and adulthood.** Quantitative RT-PCR of (A) hepatic hepcidin (B) hepatic

711   hepcidin/ iron content (C) hepatic Bmp6 and (D-F) upstream erythroid regulators of hepcidin

712   (Gdf15, Twsg1, Erfe) in the spleen of WT and BKO mice aged 6-7 weeks and 16-20 weeks.

713   Values are presented as means and SEM for minus delta Ct values (n = 5 per group). Statistical

714   analysis was performed by 2-way ANOVA with Bonferroni post-hoc test.

715

716   **Figure 5 The expression of major iron transporters in the liver and spleen of WT and BKO**

717   **mice at adolescence and adulthood.** Quantitative RT-PCR of (A) hepatic DMT1 (B) hepatic

718   ferroportin (C) splenic DMT1 and (D) splenic ferroportin of WT and BKO mice aged 6-7 weeks

719   and 16-20 weeks. Values are presented as means and SEM for minus delta Ct values (n = 5 per

720   group). Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test.

721

722   **Figure 6 The expression of major duodenal iron transport machineries in WT and BKO**

723   **mice at adolescence and adulthood.** Quantitative RT-PCR of of (A) duodenal Dcytb (B)

724   duodenal DMT1 and (C) duodenal ferroportin of WT and BKO mice aged 6-7 weeks and 16-20

725   weeks. Values are presented as means and SEM for minus delta Ct values (n = 5 per group).

726   Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test.

727

Figure 1

Figure 1 Perl's Prussian blue staining of representative paraffin-embedded tissue sections from the liver, spleen and duodenum of male WT and BKO mice aged 6-7 weeks and 16-20 weeks

The blue stain represents iron accumulation. The arrows indicate iron accumulation in brush border of enterocytes. (Nikon ECLIPSE 80i light microscope ; magnification x400)

6-7 weeks   16-20 weeks

Liver

A   B

WT

20 um   20 um

C   P   D

BKO

20 um   20 um

Spleen

E   F

WT

20 um   20 um

G   H

BKO

20 um   20 um

Duodenum

I   J

WT

20 um   20 um

K   L

BKO

20 um   20 um

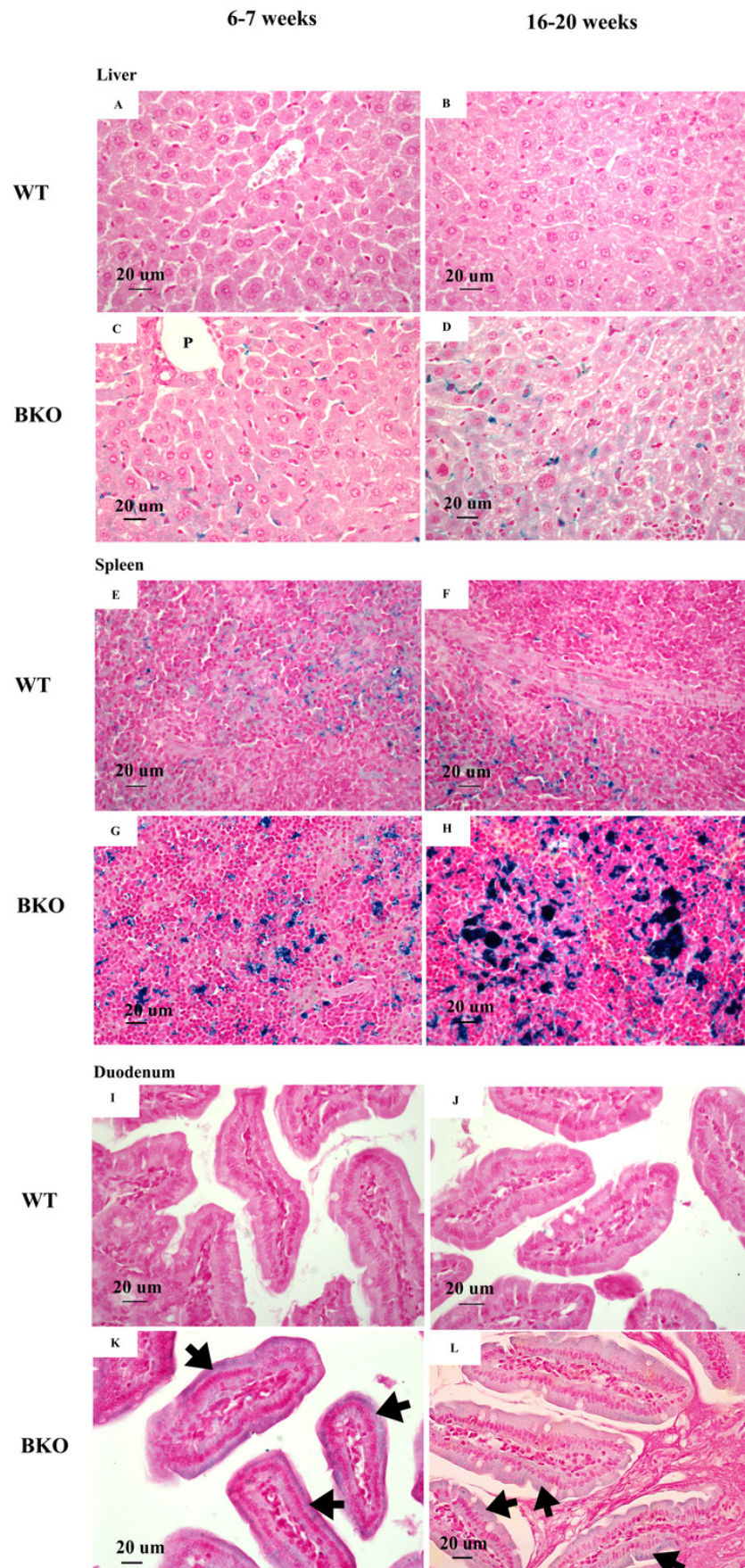



Figure 2

Figure 2 Hematoxylin and eosin staining of representative paraffin-embedded tissue sections from the liver, spleen and duodenum of male WT and BKO mice aged 6-7 weeks and 16-20 weeks

The arrows indicate mononuclear cells and hematopoietic cells in the liver and spleen. (Nikon ECLIPSE 80i light microscope ; magnification x400)

6-7 weeks   16-20 weeks

Liver

WT

BKO

Spleen

WT

BKO

Duodenum

WT

BKO

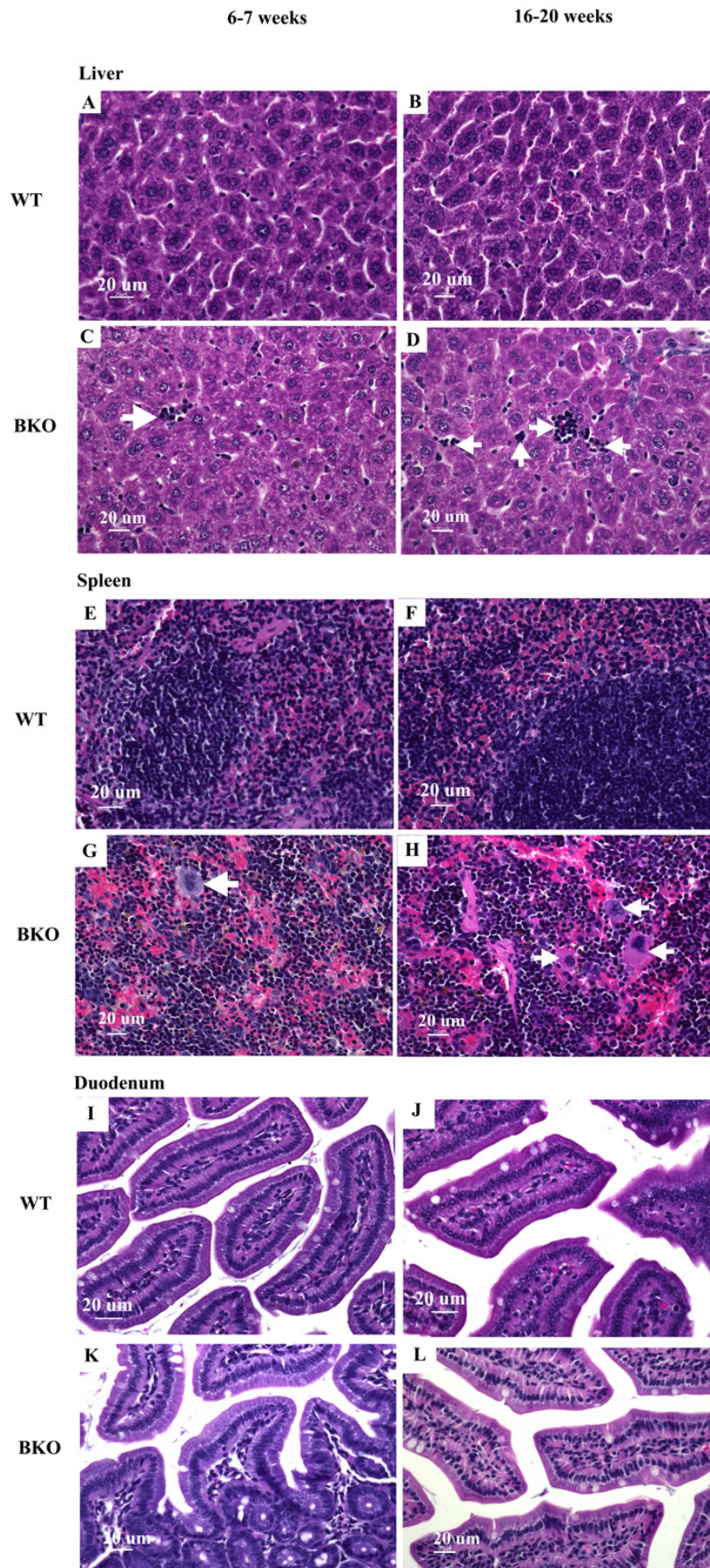



Figure 3

Figure 3 The expression of genes involved in hemoglobin biosynthesis and degradation in the spleen of wild type and thalassemic mice

Quantitative RT-PCR of *Tfrc* (TfR1), *Slc25a37* (Mfrn1), *Alas2* (ALAS2) and *Hmox1* (HO-1) mRNA from the spleen of WT and BKO mice aged 6-7 weeks and 16-20 weeks. Relative mRNA expression was acquired by normalizing to *Actb* (β-actin) mRNA. Values are presented as means and SEM for minus delta Ct values (n = 5 per group). Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test. *P*-values for the effects of phenotype, age and interaction are shown in the figure. Statistical significance for pairwise comparison is indicated by * symbols (***P* < 0.01, ****P* < 0.001)

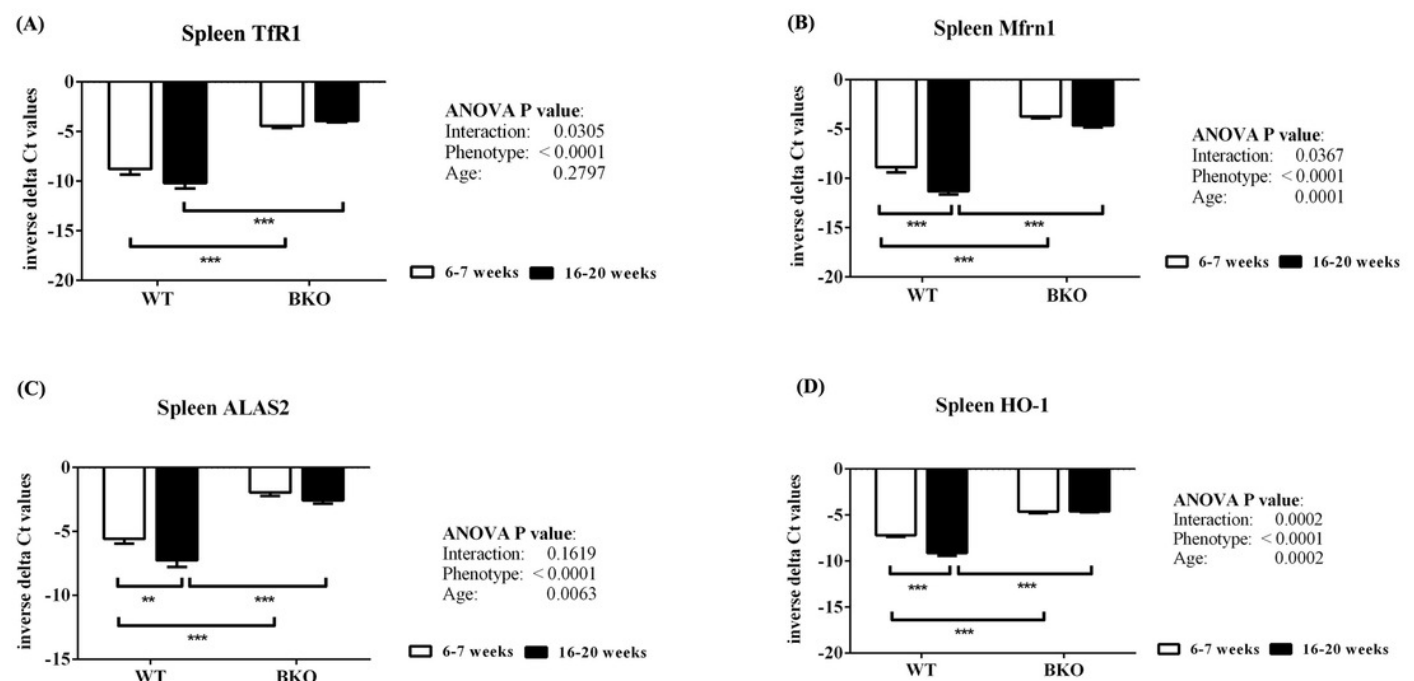



Figure 4

Figure 4 The expression of hepcidin and upstream regulators of hepcidin in wild type and thalassemic mice

Quantitative RT-PCR of (A) *Hamp* (hepcidin) mRNA in the liver (B) Liver hepcidin mRNA relative to liver iron content (C) *Bmp6* mRNA in the liver (D-F) upstream regulators of hepcidin (*Gdf15*, *Twsg1*, *Fam132b*) mRNA in the spleen of WT and BKO mice aged 6-7 weeks and 16-20 weeks. Relative mRNA expression was acquired by normalizing to *Actb* (β-actin) mRNA. Values are presented as means and SEM for minus delta Ct values (n = 5 per group). Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test. *P*-values for the effects of phenotype, age and interaction are shown in the figure. Statistical significance for pairwise comparison is indicated by * symbols (*P* < 0.05, **P* < 0.01, ***P* < 0.001)

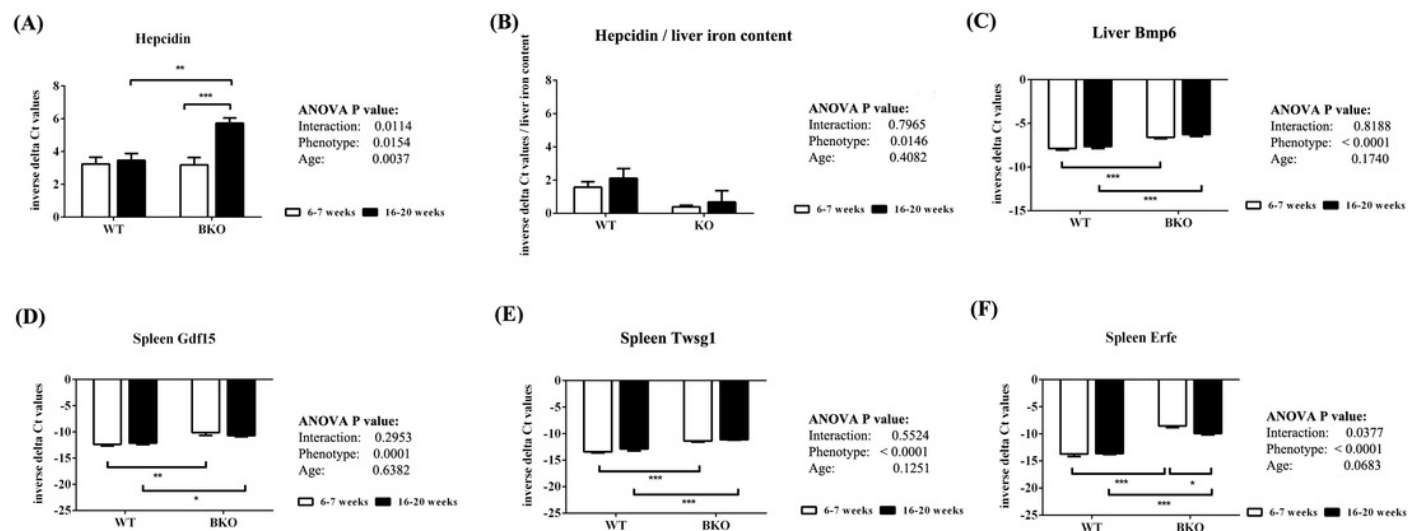



Figure 5

Figure 5 The expression of major liver and spleen iron transporters in wild type and thalassemic mice

Quantitative RT-PCR of (A) Liver *Slc11a2* (DMT1) mRNA, (B) Liver *Slc40a1* (ferroportin) mRNA (C) Spleen *Slc11a2* (DMT1) mRNA (D) Spleen *Slc40a1* (ferroportin) mRNA of WT and BKO mice aged 6-7 weeks and 16-20 weeks. Relative mRNA expression was acquired by normalizing to *Actb* (β-actin) mRNA. Values are presented as means and SEM for minus delta Ct values (n = 5 per group). Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test. *P*-values for the effects of phenotype, age and interaction are shown in the figure. Statistical significance for pairwise comparison is indicated by * symbols (****P < 0.01, ***P < 0.001)

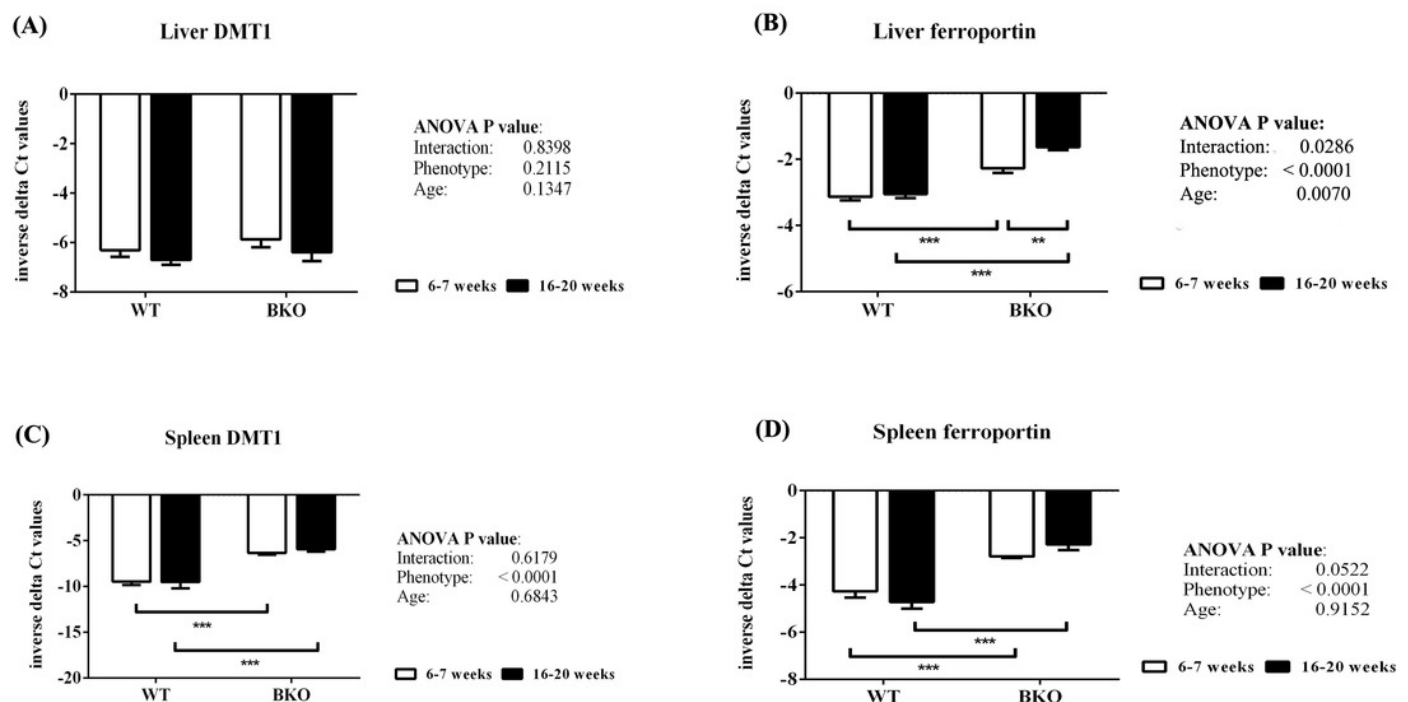



Figure 6

Figure 6 The expression of major duodenal iron transport machineries in wild type and thalassemic mice

Quantitative RT-PCR of (A) *Cybrd1* (Dcytb) (B) *Slc11a2* (DMT1) (C) *Slc40a1* (ferroportin) mRNA from the duodenum of WT and BKO mice aged 6-7 weeks and 16-20 weeks. Relative mRNA expression was acquired by normalizing to *Actb* (β-actin) mRNA. Values are presented as means and SEM for minus delta Ct values (n = 5 per group). Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test. *P*-values for the effects of phenotype, age and interaction are shown in the figure. Statistical significance for pairwise comparison is indicated by * symbols (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

(A)

(B)

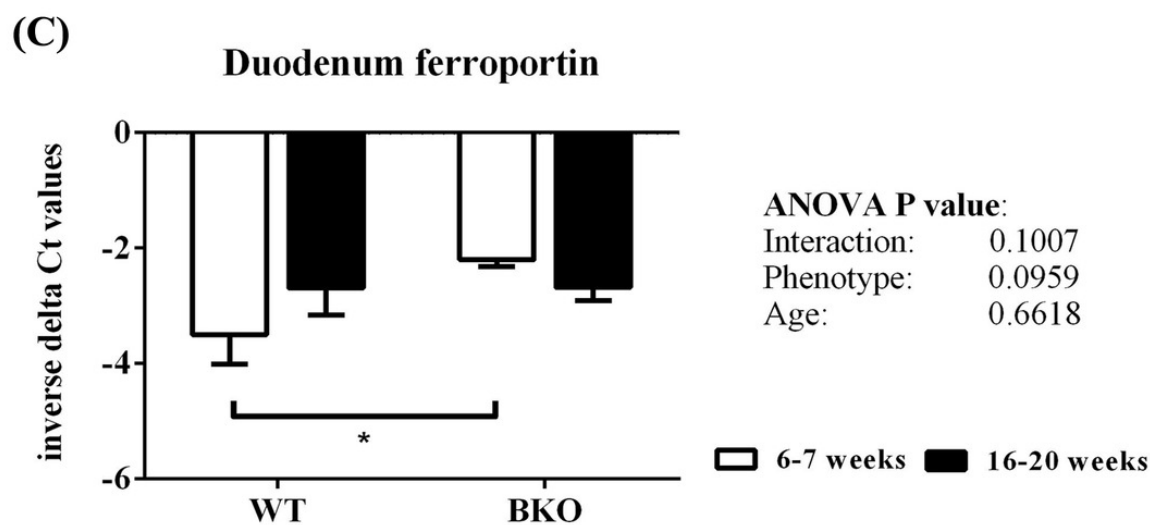
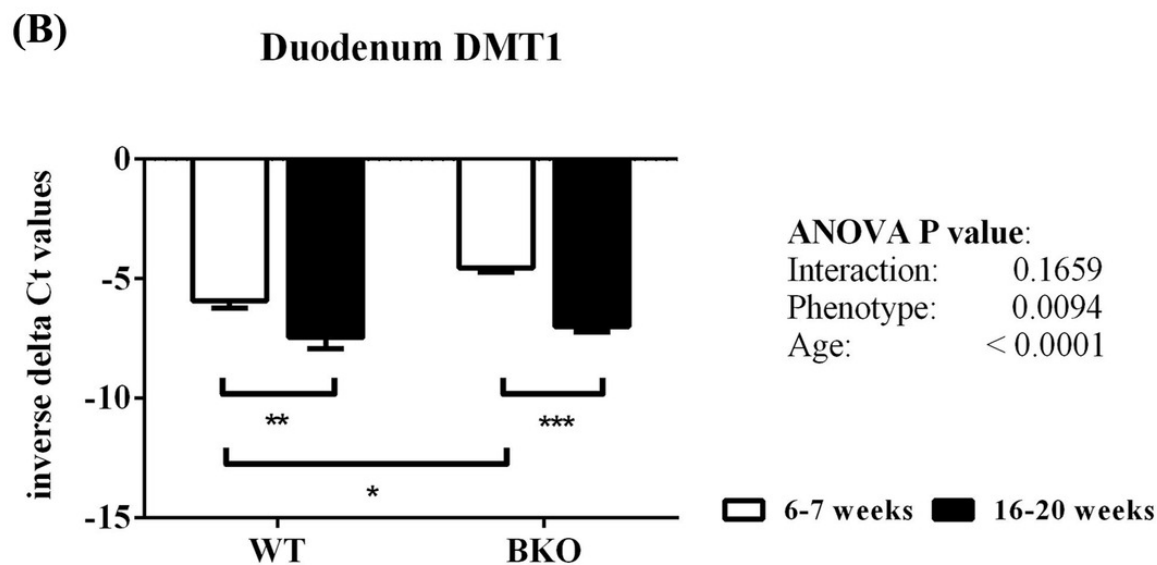
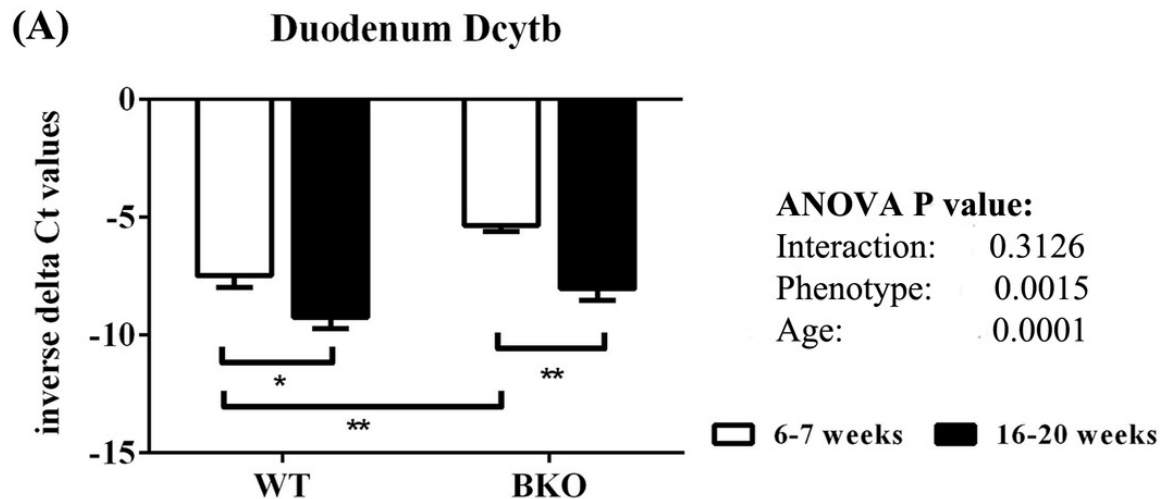
(C)

Sequence of gene-specific primers

Table 1 Sequence of gene-specific primers.

Gene product	Forward primer	Reverse primer
Actb (β-actin)	CAGCCTTCCTTCTTGGGTA	TTTACGGATGTCAACGTCACAC
Alas2 (ALAS2)	AGCCATTGTCCTTTCATGCT	CAGCAGGTCTGTCTTGAAAGTCT
Bmp6 (Bmp6)	GCCAACTACTGTGATGGAGAGTGTT	CTCGGGATTCATAAGGTGGACCA
Cybrd1 (Dcytb)	TTTGTCCTGAAACACCCCTC	AGAAGGCCCAGCGTATTTGT
Fam132b (Erfe)	TCCTCTATCTACAGGCAGGAC	ACTGCGTACCGTGAGGGA
Gdf15 (Gdf15)	GAGCTACGGGGTCGCTTC	GGGACCCCAATCTCACCT
Hamp (Hepcidin)	CAGGGCAGACATTGCGATAC	GTGGCTCTAGGCTATGTTTTGC
Hmox1 (HO-1)	CAGAGCCGTCTCGAGCATAG	CAAATCCTGGGGCATGCTGT
Slc11a2 (DMT1)	TTCTACTTGGGTTGGCAGTGTT	CAGCAGGACTTTCGAGATGC
Slc25a37 (Mfrn1)	ACGCCATGTATTTTGCCTGC	ACTCCCAGCTACCCCATTAG
Slc40a1 (ferroportin)	ATCCCCATAGTCTCTGTCAGC	CAGCAACTGTGTCACCGTCA
Tfrc (TfR1)	TCCTTTCCTTGCATATTCTGG	CCAAATAAGGATAGTCTGCATCC
Twsg1 (Twsg1)	GCTGTCACACCATGAAAACCTAG	ACTGTGCACATGCGCTCTT

2

Hematological data and iron parameters of male wild type and thalassemic mice at the age of 6-7 weeks and 16-20 weeks

Table 2 Hematological data and iron status in various ages of male WT and BKO mice.

	WT		BKO		P values (2-way ANOVA)		
	6-7 weeks	16-20 weeks	6-7 weeks	16-20 weeks	Age	Phenotype	Age x Phenotype
RBC count (10^6/µl)	6.25 ± 0.45	8.91 ± 0.35 [a]	4.95 ± 0.45	4.68 ± 0.66 [b]	0.0270	< 0.0001	0.0087
Hemoglobin (g/dL)	9.84 ± 0.66	14.38 ± 0.65 [a]	6.46 ± 0.10 [a]	5.68 ± 0.90 [b]	0.0104	< 0.0001	0.0008
Hematocrit (%)	35.42 ± 1.98	44.66 ± 1.40 [a]	22.72 ± 1.70 [a]	19.18 ± 2.54 [b]	0.1634	< 0.0001	0.0048
MCV (fL)	57.06 ± 1.71	50.16 ± 0.47 [a]	46.38 ± 1.51 [a]	41.40 ± 1.87 [b]	0.0011	< 0.0001	0.5295
MCH (pg)	15.82 ± 0.65	16.12 ± 0.15	13.58 ± 1.52	12.24 ± 1.25 [b]	0.6235	0.0095	0.4415
MCHC (g/dL)	27.78 ± 1.10	32.14 ± 0.52	29.04 ± 2.24	29.34 ± 1.79	0.1541	0.6277	0.2109
RDW (%)	23.02 ± 1.62	12.88 ± 0.05 [a]	34.62 ± 2.69 [a]	27.28 ± 3.28 [b]	0.0014	< 0.0001	0.5462
Serum iron (µM)	53.21 ± 3.55	18.47 ± 1.69 [a]	44.44 ± 2.44	20.49 ± 3.27 [c]	< 0.0001	0.2509	0.0750
Liver non-heme iron (nmole/mg wet weight)	2.30 ± 0.34	1.92 ± 0.27	9.05 ± 1.67 [a]	8.72 ± 0.91 [b]	0.7207	< 0.0001	0.9799
Spleen non-heme iron (nmole/mg wet weight)	5.37 ± 0.40	7.50 ± 0.48	18.97 ± 1.33 [a]	59.22 ± 5.72 [b,c]	< 0.0001	< 0.0001	< 0.0001

Notes.

RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

Data expressed as mean ± SEM (n = 5/group). Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test.

a, b: P-value < 0.05 compared with WT aged 6-7 weeks and 16-20 weeks, respectively.

c: P-value < 0.05 compared with BKO aged 6-7 weeks.

Iron deposition score in the liver, spleen and duodenum of male wild type and thalassemic mice at the age of 6-7 weeks and 16-20 weeks

Table 3 Iron deposition score in the liver, spleen and duodenum of WT and BKO mice at adolescence (6-7 weeks old) and adulthood (16-20 weeks old).

Age	Iron deposition score		
	Liver	Spleen	Duodenum
WT			
6 - 7 weeks	0	1	0
16 - 20 weeks	0	1	0
BKO			
6 - 7 weeks	1	2	1
16 - 20 weeks	1	3	1

3   The same deposition score was obtained from the mouse within same group (n = 5 per group).

Mononuclear cells infiltration and hematopoietic cells score in the liver of male wild type and thalassemic mice at the age of 6-7 weeks and 16-20 weeks

Table 4 Mononuclear cells infiltration and hematopoietic cells score in the liver of male WT and thalassemic mice at different age ranges.

	WT		BKO		*P* values (2-way ANOVA)		
	6-7 weeks	16-20 weeks	6-7 weeks	16-20 weeks	Age	Phenotype	Age x Phenotype
Mononuclear cells and hematopoietic cells score	0.12 ± 0.02	0.16 ± 0.14	1.86 ± 0.08 [a]	2.92 ± 0.15 [b,c]	0.0001	< 0.0001	0.0003

2 **Notes.**

3 Data expressed as mean $\pm$ SEM (n = 5/group). Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test.

4 a, b : *P*-value < 0.05 compared with WT aged 6-7 weeks and 16-20 weeks, respectively.

5 c : *P*-value < 0.05 compared with BKO aged 6-7 weeks