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Transgenic HFE-dependent induction of hepcidin in mice does not require transferrin receptor-2

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Abstract

Hereditary hemochomatosis (HH) is caused by mutations in several genes, including *HFE* and transferrin receptor-2 (*TFR2)*. Loss of either protein decreases expression of the iron regulatory hormone hepcidin by the liver, leading to inappropriately high iron uptake from the diet, and resulting in systemic iron overload. In tissue culture, overexpressed HFE and TFR2 physically interact. Hepatocellular overexpression of Hfe in vivo increases hepcidin expression, despite an associated decrease in Tfr2. On this basis, we hypothesized that Tfr2 would not be required for Hfe-dependent up-regulation of hepcidin. We show that hepatocellular overexpression of Hfe in $Tfr2^{Y245X/Y245X}$ mice leads to hepcidin induction eventuating in iron deficiency and a hypochromic, microcytic anemia. Furthermore, co-immunoprecipitation studies using liver lysates did not provide evidence for physical interaction between Hfe and Tfr2 in vivo. In conclusion, we demonstrate that Tfr2 is not essential for Hfe-mediated induction of hepcidin expression, supporting the possibility that TFR2 may regulate iron metabolism in an HFE-independent manner.

Keywords

Hfe; Tfr2; hemochromatosis

Introduction

Iron is essential for a wide variety of metabolic processes, but is highly toxic in excess. As a result, systemic iron homeostasis is tightly regulated. Hereditary hemochromatosis (HH) is a common disease caused by a chronic inappropriate increase in dietary iron uptake, leading to iron overload over a period of years. HH is most often caused by recessive mutations in *HFE* [1]. Less often, autosomal recessive mutations in hemojuvelin (*HJV*) [2], hepcidin (*HAMP*) [3] and transferrin receptor-2 (*TFR2*) [4], as well as dominant mutations in ferroportin (*SLC40a1*) [5], also cause HH. Each of these mutations disrupts the regulated synthesis or function of hepcidin, a peptide hormone largely produced by the liver, which promotes the degradation of ferroportin, an iron exporter present on the surface of macrophages of the reticuloendothelial system as well as duodenal enterocytes [2, 3, 6–10].

HFE is an atypical major histocompatibility (MHC) class I-like molecule [11] that associates with both β2-microglobulin (β2M) [12] and transferrin receptor-1 (*TFR1*) [13, 14]. HFE

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Authorship and Disclosures

P.J.S. conceived and designed the murine experiments, analyzed the data, and wrote the manuscript; M.D.F oversaw the research and assisted in writing of the manuscript. The authors report no conflicts of interest.

functions in the liver to regulate hepcidin, and mice lacking Hfe only in hepatocytes have an HH phenotype [15]. TFR2 is a homolog of TFR1 expressed predominately in the liver [16] that does not clearly have a role in cellular iron uptake. Among other alleles, the truncation mutation Y250X causes HH in humans, and a murine model with the equivalent Y245X mutation has an HH phenotype and inappropriately low hepatocyte hepcidin expression [17, 18].

We showed that transgenic mice carrying mutations in Tfr1 that interfere with its interaction with Hfe have decreased hepcidin expression [19]. Although initial studies with soluble proteins detected no interaction between HFE and TFR2 by co-immunoprecipitation or surface plasmon resonance analysis [20], work in transient transfection cell culture systems has indicated that Hfe also interacts with Tfr2 and that an Hfe-Tfr2 complex is favored over the Hfe-Tfr1 interaction when high levels of differic transferrin (Fe_2-Tf) are present [21]. On this basis we, and others, have hypothesized that competition between Tfr1 and Tfr2 for Hfe binding allows hepcidin to be regulated in response to the amount of circulating Fe_2-Tf [19, 21, 22]. A report that constitutive viral overexpression of murine Hfe in hepatocytes of $Tfr2^{Y245X/Y245X}$ animals does not lead to decreased non-heme liver iron, transferrin saturation or elevated hepcidin expression supports the notion that HFE requires TFR2 to regulate iron metabolism [23]. However, other results suggest that the effect of HFE and TFR2 on hepcidin expression may not be entirely co-dependent. For example, we showed that transgenic overexpression of Hfe increases hepcidin mRNA and induces iron deficiency while simultaneously down-regulating Tfr2 [24]. Furthermore, humans and mice with loss of function mutations in both *HFE* and *TFR2* have a clinically more severe phenotype than individuals with either mutation alone [25, 26].

To further interrogate the role of TFR2 in HFE-dependent regulation of hepcidin *in vivo*, we bred the previously described Hfe^{WT} [19], and $Hfe^{\Delta CD\text{-}MYC}$ [24] transgenic lines to animals lacking full-length Tfr2. Our results indicate that Tfr2 is not required for Hfe-mediated regulation of hepcidin. Moreover, we were not able to confirm Tfr2-Hfe interaction in this *in vivo* system.

Design and Methods

Generation of transgenic mice expressing modified *Hfe* under control of the *transthyretin* (*TTR*) promoter

A full-length mouse Hfe transgene (Hfe^{WT}) under the control of the transthyretin (TTR) promoter (pPJS095) has been described previously [19] and is available from the Mouse Mutant Resource Center (MMRC, #030621-UNC). A truncated murine Hfe transgene with a cMyc epitope replacing the final 15 amino acids ($Hfe^{\Delta CD\text{-}MYC}$) of the protein has also been described [24]. Mice expressing the full length Hfe^{WT} or $Hfe^{\Delta CD\text{-}MYC}$ truncated transgene on a C57BL6/J background were bred to $Tfr2^{Y245X/Y245X}$ animals (kind gift of Robert Fleming) on an FVB/J background. All mice analyzed in this cohort were littermates on a mixed C57BL6/J-FVB/J background. Mice on a C57BL/6J background were bred with $\beta 2m^{-/-}$ mice on the same genetic background to generate $\beta 2m^{-/-}$ animals carrying a TTR-Hfe transgene (Hfe^{WT} or $Hfe^{\Delta CD\text{-}MYC}$). All Hfe transgenic animals were hemizygous for the transgene.

Oligonucleotide primers

Oligonucleotide primers employed in PCR genotyping and for quantitative PCR analysis are listed in a Supplementary Table I.

Animal care and analysis

All genetically modified mice were born and housed in the barrier facility at Children's Hospital Boston and handled according to protocols approved by the Institutional Animal Care and Use Committee. Animals were maintained on the Prolab RMH 3000 diet (Lab Diet, 380ppm iron). The facility employs a constant dark-night light cycle, and all animals were provided both water and food *ad libitum*. Due to differences in iron metabolism between male and female animals, only females were analyzed. All animals were killed and analyzed at 8-weeks of age.

PCR genotyping

PureLink Genomic DNA Mini Kits (Invitrogen) were used to prepare genomic DNA from tail snips. Transgenic *TTR-Hfe* mice were genotyped by PCR with primers PS-133 and PS-134 to yield a 450bp band. $Tfr2^{Y245X/Y245X}$ mice were genotyped by PCR using primers TFR2 Y245XF and TFR2 Y245XR yielding an 814bp WT band and a 922bp KO band. $\beta 2m^{-/-}$ mice were genotyped using primers IMR0160, IMR0184 or IMR0185 yielding a 261bp WT band or 410bp KO band (Supplementary Table I).

Immunohistochemisty

Liver tissue samples were fixed in 10% buffered formalin for 24 hours, transferred to 70% ethanol and then embedded in paraffin. Formalin-fixed paraffin embedded tissue sections were mounted on microscope slides. Immunohistochemical staining for c-Myc (rabbit anti-cMyc, Abcam) was performed using a Ventana Discovery XT automated immunohistochemistry slide processing platform as described previously [24] at the Children's Hospital Boston Pathology Research Core Laboratory. Images were acquired using a 40X/0.75 objective lens and 2X camera adaptor (final magnification of 80X) of a BX50 microscope with DP25 Digital Camera employing DP2-BSW software (Olympus).

Blood and tissue iron analysis

Whole blood for complete blood counts was collected retro-orbitally into EDTA-coated microtainer tubes (Becton Dickinson) from animals anesthetized with 2.5% tribromoethanol in isoamyl alcohol (Avertin). Samples were analyzed on an Avida 120 analyzer (Bayer) by the Children's Hospital Boston Department of Laboratory Medicine Clinical Core Laboratories. Whole blood for other purposes was collected by retro-orbital bleeding into serum separator tubes (Becton Dickinson), and serum was prepared according to the manufacturer's instructions. Serum iron values were determined with the Serum Iron/UIBC kit (Thermo Fisher) according to the manufacturer's instructions. Liver and spleen tissues were collected and tissue non-heme iron concentrations were determined as described previously [27].

RNA extraction, RT-PCR, semi-quantitative and quantitative PCR

Total liver RNA was isolated from flash-frozen tissue in TRIzol (Invitrogen). Total RNA was treated with DNase I (Roche) to remove contaminating genomic DNA. cDNA was synthesized from the resulting RNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time PCR quantification of hepcidin (*Hamp*), *β-actin* and *Bmp6* mRNA transcript levels was performed as described previously [24]. All primers are listed in Supplementary Table I.

Immunoprecipitation from mouse tissue lysates

Liver samples were flash frozen in liquid nitrogen and manually lysed in NET buffer (150 mM NaCl, 50mM Tris-HCl pH 7.5, 2mM EDTA, 1%Triton X-100) and cell debris removed by centrifugation. A total of 1 mg of total cell lysate was brought up to 250 μl in NET buffer

and pre-adsorbed with 35 μl TrueBlot Anti-Rabbit Ig IP Beads (eBioscience) for 1 hour. Preabsorbed lysates were incubated overnight with 5 mg rabbit anti-c-Myc (Abcam). TrueBlot Anti-Rabbit Ig IP Beads (50 μl) was added and lysates were incubated for 1 hour tumbling. The Sepharose pellet was washed 3 times with NET buffer and samples were eluted in 75 μl 2X Laemmli loading buffer (100mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol) with a final concentration of 0.2 M dithiothreitol.

Immunoblot analysis

Tissues were manually lysed in NET buffer (150 mM NaCl, 50mM Tris-HCl pH 7.5, 2mM EDTA, 1%Triton X-100) supplemented with Complete Mini, EDTA-free protease inhibitor cocktail tablets (Roche). Cell debris was removed by centrifugation. 150 μg of total liver tissue protein diluted in 2X Laemmli loading buffer (0.2 M DTT final) or 35μl of IP re-suspension, were boiled and electrophoresed on precast 4–12% or 10% polyacrylamide NuPage (Invitrogen) gels. Proteins were transferred onto nitrocellulose membranes and immunoblot analysis was performed using rabbit anti-cMyc (1:2500, Abcam), rabbit anti-mouse Tfr2 (1:500, Alpha Diagnostic International, Inc), rabbit anti-β-actin (1:1000, Cell Signaling), mouse anti-Tfr1 (1:500, Invitrogen) or rabbit anti-β2m (1:5000, Abcam). Blots were then incubated with anti-rabbit or anti-mouse (1:5000) secondary antibody conjugated to horseradish peroxidase and then subjected to chemiluminescence (Amersham, ECL) per the manufacturer's directions. Between immunoblot analyses, blots were stripped with ReBlot Strong (Chemicon).

Statistics

A two-tailed Student's *t*-test (Microsoft Excel) with $P<0.05$ was employed to determine statistical significance.

Results

Loss of full-length Tfr2 does not affect transgenic Hfe-mediated induction of hepcidin expression

Previous *in vitro* work indicated that HFE and TFR2 physically interact [21] and that contact between the two proteins is essential for transferrin-induced regulation of hepcidin [22]. Mice with a $Tfr2^{Y245X/Y245X}$ targeted mutation, orthologous to the Y250X disease-causing allele in humans [4], have greatly elevated liver and decreased splenic non-heme iron, normal hematopoietic parameters [17], and diminished hepcidin expression [18]. Using an adenoviral system, Gao *et al.* found that transgenic expression of Hfe in the liver of $Tfr2^{Y245X/Y245X}$ animals does not alter the HH phenotype [23]. We, however, recently showed that Hfe transgene-induced hepcidin expression even as Tfr2 is down-regulated [24]. Thus, in order to further examine whether HFE-dependent hepcidin expression requires TFR2 *in vivo*, we bred $Tfr2^{Y245X/Y245X}$ animals to liver-specific *Hfe*-transgenic mice.

$Tfr2^{Y245X/Y245X}$ animals have elevated liver non-heme iron, transferrin saturation and serum iron, and diminished spleen non-heme iron (Fig. 1A–D). We found that expression of the Hfe^{WT}-transgene overcorrects the $Tfr2^{Y245X/Y245X}$ iron loading phenotype, and causes iron deficiency (Fig. 1A–D). Interestingly, however, even though Hfe^{WT} $Tfr2^{Y245X/Y245X}$ mice have greatly decreased tissue and serum iron levels equivalent to their wild type transgenic (Hfe^{WT} $Tfr2^{+/+}$) littermates they do not develop as severe an anemia (Table I). However, $Tfr2^{Y245X/Y245X}$ animals that carry the $Hfe^{\Delta CD\text{-}MYC}$ epitope-tagged transgene do have a significant hypochromic, microcytic anemia (Supplementary Table II). The cause of this difference is likely due to the fact that Hfe protein expression is much higher in $Hfe^{\Delta CD\text{-}MYC}$ compared to Hfe^{WT} transgenic mice [24]. Regardless, Hfe^{WT} $Tfr2^{Y245X/Y245X}$

mice are iron-poor compared to WT or *Tfr2*$^{Y245X/Y245X}$ animals, indicating that Tfr2 is not essential for the effect of the Hfe transgene on iron metabolism.

Liver hepcidin mRNA expression is decreased relative to liver non-heme iron content in *Tfr2*$^{Y245X/Y245X}$ mice [18]. In contrast, hepcidin levels are inappropriately high for liver iron stores in all *Hfe*^{WT}-transgenic animals (Fig. 2A). It has been shown that *Bmp6* mRNA levels are increased in response to increased iron stores in both dietary iron overload and the Tfr2 knockout animal [28, 29]. Similarly, we observed an increase in *Bmp6* mRNA levels in *Tfr2*$^{Y245X/Y245X}$ mice (Fig. 2B), and found that *Hfe*^{WT}-transgenic animals have a significant diminution of *Bmp6* mRNA expression compared to both WT and *Tfr2*$^{Y245X/Y245X}$ mice (Fig. 2B). Both relative hepcidin and BMP6 mRNA expression, when compared to non-heme liver iron (Fig. 2C and D, respectively), are greatly increased in all iron-poor *Hfe*^{WT}-transgene containing animals and both are inappropriately low in *Tfr2*$^{Y245X/Y245X}$ animals when normalized for iron stores.

β2M is essential for HFE-mediated induction of hepcidin expression

In cell culture, the common HH HFE C282Y mutation abrogates interaction with β2M, causing mislocalization of HFE [12, 30]. Loss of Hfe or β2m in live mice leads to dysregulation of hepcidin expression [10, 31]. We previously demonstrated that hepatocyte-specific transgenic expression of either WT *Hfe* cDNA (*Hfe*^{WT}), or a chimeric Hfe containing a cMyc epitope tag in place of the cytoplasmic domain (*Hfe*$^{\Delta CD\text{-}MYC}$) prevented iron overload in *Hfe*$^{-/-}$ animals, and even resulted in iron deficiency attributable to hepcidin dysregulation [19, 24]. To confirm that β2M is necessary for the HFE-mediated regulation of hepcidin *in vivo*, and to demonstrate that overexpression of Hfe is unable to increase hepcidin expression in the absence of a protein known to be required for Hfe function, we bred *Hfe*^{WT} trangenic mice to animals lacking β2m.

We compared the phenotypes of *β2m*$^{-/-}$ animals with and without the liver-specific *Hfe*^{WT} transgene. We found that the *Hfe*^{WT} transgene was unable to correct the *β2m*$^{-/-}$ iron loading phenotype (Supplementary Fig. 1A–D); animals lacking β2m have elevated non-heme liver iron stores and decreased non-heme spleen iron stores regardless of Hfe-transgene status (Supplementary Fig. 1A–B). Furthermore, the transferrin saturation and the serum iron concentration are both increased in *β2m*$^{-/-}$ animals whether or not they carry the *Hfe*^{WT} transgene (Supplementary Fig. 1C–D), whereas *Hfe*^{WT} overexpression in WT animals causes decreased serum iron parameters (Supplementary Fig. 1C–D). Likewise, *β2m*$^{-/-}$ or *β2m*$^{-/-}$ Hfe-transgenic positive animals have hematologic parameters comparable their WT littermates, but, as reported previously [32], *Hfe*^{WT} transgenic animals have a hypochromic, microcytic anemia (Supplementary Table III). *β2m*$^{-/-}$ mice with or without the *Hfe*^{WT} transgene express less hepcidin, and more Bmp6 mRNA, than WT animals (Supplementary Fig. 2A–B). However, WT animals expressing the *Hfe*^{WT}-transgene have significantly elevated hepcidin and decreased Bmp6 mRNA (Supplementary Fig. 2A–B), providing an explanation for the severe anemia observed in these animals (Supplementary Fig. 1A–D and Supplementary Table III). Correspondingly, when compared to non-heme liver iron content, hepcidin and Bmp6 mRNA levels are inappropriately low in all *β2m*$^{-/-}$ mice and greatly elevated in *Hfe*^{WT} transgenic animals (Supplementary Fig. 2C–D). These results confirm that β2m is essential for the Hfe-mediated induction of hepcidin *in vivo* and suggest that induction of hepcidin by the transgenically expressed Hfe occurs through a biologically relevant pathway.

β2M, but not full-length TFR2, is necessary for the proper localization of HFE to the cell membrane

Mutations in human HFE abrogate the interaction between HFE and β2M causing the mislocalization of HFE [12, 30]. Mutant HFE protein is retained in the endoplasmic reticulum and middle Golgi compartment and is subject to accelerated degradation [30]. However, all of these mechanisms have been demonstrated in cell culture systems. In order to confirm that loss of β2M, and determine if loss of full-length TFR2, leads to mislocalization of HFE, we employed immunohistohemistry (IHC) to visualize transgenically expressed Hfe protein in the liver. It is not possible to visualize expression of the Hfe^{WT} protein product by IHC with the available antibodies. To circumvent this problem, we employed the c-Myc epitope tagged Hfe transgenic line, $Hfe^{\Delta CD\text{-}Myc}$ [24]. We bred $Hfe^{\Delta CD\text{-}Myc}$ animals to both $\beta 2m^{-/-}$ and $Tfr2^{Y245X/Y245X}$ mice and analyzed the offspring. Animals expressing the $Hfe^{\Delta CD\text{-}Myc}$ transgene on either the $\beta 2m^{-/-}$ or $Tfr2^{Y245X/Y245X}$ background are phenotypically qualitatively similar (Supplementary Fig. 3A–D, Supplementary Tables 2 and 4) from mice of comparable genotypes expressing the Hfe^{WT} transgene (Fig. 1A–D, Supplementary Fig. 1A–D, Table 1 and Supplementary Table III).

Using IHC, we demonstrate that the $Hfe^{\Delta CD\text{-}Myc}$ transgene protein product is localized to the cell membrane, regardless of Tfr2 status (Fig. 3A–C). Conversely, $\beta 2m^{-/-}$ animals are unable to properly localize Hfe-cMyc to the membrane of hepatocytes (Fig. 3D–F). In fact, the protein is found within the cell, primarily in a pattern consistent with the Golgi and ER compartments. This confirms that β2M is essential, but full-length TFR2 is not required, for proper localization of HFE to the cell membrane. This reaffirms that loss of the HFE-β2M interaction leads to a manifestation of HH and iron overload as previously noted in human patients with mutations in *HFE* [12].

Overexpression of Hfe does not directly affect expression of β2m, Tfr1 or Tfr2 or preclude a β2m-Hfe interaction

It is possible that overexpression of transgenic-Hfe leads to the altered expression levels of β2m, Tfr1 or Tfr2. Diminution of β2m protein could lead to the observed decrease in Hfe^{WT} and $Hfe^{\Delta CD\text{-}Myc}$ function (Supplementary Fig. 1A–D and Supplementary Fig. 2A–D). We employed Western Blot and immunoprecipitation (IP) analysis to better understand the function of transgenically expressed Hfe *in vivo*.

Animals carrying the $Hfe^{\Delta CD\text{-}Myc}$ transgene have decreased Tfr2 expression (Fig. 4A top panel). This was expected due to the decreased Tf saturation (Supplementary Fig. 3D) of the animals, as an elevated Tf saturation is known to stabilize TFR2 expression [33, 34]. Previously we found that endogenous Tfr2 expression in $Hfe^{\Delta CD\text{-}Myc}$ animals was greatly decreased [24]; however, here we are better able to visualize Tfr2 protein upon overexpression of Hfe. This may be due to either the mixed C57Bl/6J and FVB background of the animals or the use of an increased titer of antibody. Notably, although the Tfr2 antibody recognizes N-terminal domain of the protein, no truncated, soluble $Tfr2^{Y245X}$ protein is detected, consistent with previous observations [17], suggesting that the mRNA is subject to nonsense mediated decay or that the mutated protein is degraded. Conversely, Tfr1, which is known to be inversely regulated by intracellular iron levels, is appropriately upregulated in anemic $Hfe^{\Delta CD\text{-}Myc}$ animals and nearly absent in iron overloaded $Tfr2^{Y245X/Y245X}$ animals (Fig. 4A, third panel). Importantly, expression of the $Hfe^{\Delta CD\text{-}Myc}$ protein does not affect the expression of β2m (Fig. 5A, top panel).

In vitro, HFE and β2M proteins interact and it has been postulated that this complex is necessary for proper HFE localization and function [12, 30]. Likewise, it has been

demonstrated that HFE and TFR2 interact in tissue culture models of overexpressed proteins [21] most likely through the α3-domain of HFE [22]. However, neither complex has been demonstrated *in vivo*. Furthermore, HFE and TFR1 proteins interact [13, 14] and we previously showed that Tfr1 sequesters Hfe, preventing Hfe-mediated hepcidin induction in the liver [19]. We immunoprecipitated $Hfe^{\Delta CD\text{-}Myc}$ from liver lysates to interrogate these protein-protein interactions *in vivo*. $Hfe^{\Delta CD\text{-}Myc}$ was able to co-immunoprecipitate Tfr1 (Fig. 4B, bottom panel) and β2m (Fig. 5B, middle panel) demonstrating that loss of the cytoplasmic domain did not produce a misfolded chimeric protein. Furthermore, loss of Tfr2 expression in $Tfr2^{Y245X/Y245X}$ animals does not prevent an Hfe-β2m interaction (Fig. 4B, third panel). Conversely, lack of β2m expression in $\beta 2m^{-/-}$ mice does not facilitate an Hfe-Tfr2 interaction (Fig. 5B, bottom panel). Nonetheless, we were unable to detect an interaction between the $Hfe^{\Delta CD\text{-}Myc}$ protein and Tfr2 in the presence (Fig. 4B, second panel) or absence (Fig. 5B, bottom panel) ofβ2m. Immunoprecipitation with antibodies recognizing Tfr2 were also unable to detect an Hfe-Tfr2 interaction (data not shown).

Discussion

HFE, TFR2 and β2M are necessary for the normal regulation of hepcidin production in hepatocytes [9, 10, 31, 35, 36] but the role each plays is not completely understood. We sought to determine if TFR2 is necessary for HFE-dependent induction of hepcidin and to understand the functional relationship between the two proteins. To do so, we bred mice overexpressing Hfe in the liver to animals lacking Tfr2. We determined that Tfr2 is not required for Hfe-mediated induction of hepatic hepcidin. Our results also reaffirm the finding that loss of the β2M-HFE association causes mislocalization of HFE *in vitro* [12], leading to inadequate hepcidin expression and systemic iron overload. Although Hfe clearly interacts with bothβ2m and Tfr1, we were unable to visualize an Hfe-Tfr2 complex as previously demonstrated in tissue culture [21, 22].

These studies expand our knowledge of HFE-dependent pathways of hepcidin induction. Earlier work utilizing tissue culture models [21, 22] or viral expression of Tfr2 or Hfe in mouse liver [23] indicated that TFR2 was required for HFE-mediated hepcidin induction. We found, however, that germline transgenic overexpression of Hfe is able to complement the hepcidin deficiency and iron overload phenotype of the $Tfr2^{Y245X/Y245X}$ animal model, and that, while transgenically expressed Hfe with a cMyc epitope tag substituted for its cytoplasmic domain interacts with β2m and Tfr1, it does not interact with Tfr2 *in vivo.* The cause of these distinctly different results between model systems is not readily apparent. However, we can speculate that the germline Hfe transgenic models we employed allow for elevated expression of the protein over the lifetime of the animal, permitting the effect of Hfe expression to accrue over time. By contrast, the viral Hfe expression construct employed by Gao *et al.* was injected 8 weeks after birth and allowed to express protein for two weeks [23]. It is possible that Tfr2 is only necessary for, or has a more pronounced effect on, hepcidin induction in young animals. Alternately, the effect could be a result of the relatively higher levels of Hfe protein expression in the transgenic Hfe^{WT} compared to the viral infection model [23, 24]; high HFE protein levels could stimulate an alternate pathway of HFE-dependent hepcidin expression that is independent of TFR2. Finally, we have demonstrated that Hfe overexpression in Hfe^{WT} or Hfe^{WT} $Tfr2^{Y254X/Y254X}$ animals leads to diminished iron stores and inappropriately high *Bmp6* and *hepcidin* mRNA expression in comparison to non-heme liver iron concentrations. In contrast, $Hfe^{-/-}$ [24, 32, 37, 38] and $Tfr2^{Y254X/Y254X}$ or $Tfr2^{-/-}$ animals (Fig. 2D and [29, 38]) have inappropriately low *Bmp6* and *hepcidin* mRNA when normalized for elevated iron stores. It has been postulated that HFE and TFR2 function to directly modulate the expression of hepcidin through the BMP/HJV signaling cascade. From the data presented here, it is not possible to determine if either protein regulates hepcidin or BMP6 expression in series with, or parallel

to, BMP/HJV-dependent signaling. Although it is likely that both proteins converge, at least indirectly, on the BMP-mediated signal transduction cascade, whether or not HFE or TFR2 function prior to, or after, BMP6 mRNA regulation remains to be determined.

Earlier work indicated that HFE interacts with TFR2. In cell culture, the HFE-TFR2 interaction was favored under conditions where the TF saturation is increased [21, 22]. As such, it was proposed that the competition between TFR1 and TFR2 for HFE binding allows hepcidin expression to be regulated in response to the concentration of Fe_2-TF [19, 21, 22]. Here, although we are able to demonstrate by co-immunoprecipitation studies that a germline, transgenically-expressed Hfe-cMyc chimeric protein is able to form a complex with either β2m or Tfr1, confirming earlier data [1, 12, 13], we were unable to confirm in vivo the Hfe-Tfr2 interaction reported in cell culture systems. There are several possible explanations for this apparent discrepancy. One possibility is that the cytoplasmic domain of Hfe, which is replaced by a cMyc epitope in the $Hfe^{\Delta CD\text{-}MYC}$ mouse, may be required for HFE-TFR2 heterodimer formation. However, $Hfe^{\Delta CD\text{-}MYC}$ $Tfr2^{Y245X/Y245X}$ animals have reduced iron stores and elevated hepcidin levels, indicating that the truncated Hfe is functional even if it cannot associate with Tfr2. Moreover, the Hfe-cMyc protein chimera contains the α3 domain previously postulated to be the portion of the molecule critical for the HFE-TFR2 interaction [22]. Furthermore, it is possible that overexpression of Hfe prevents Tfr2 targeting to the plasma membrane. However, we have demonstrated that the Hfe-cMyc protein forms a complex with Tfr1 suggesting that overexpression of Hfe does not affect the proper trafficking and function of a homologous membrane protein. Also, prior experiments in cell culture systems demonstrating either direct or functional HFE-TFR2 interactions overexpressed both HFE and TFR2 [21, 39] or HFE alone [22]. In addition, perhaps co-immunoprecipitation studies using lysate from a pure cell population permits identification of an HFE-TfR2 complex that is disrupted by a factor in tissue lysates. Finally, it is possible that HFE and TFR2 work in parallel to sense serum iron levels and control hepcidin expression. In addition to our results, the observation that humans [25] and mice [26] with mutations in both *HFE* and *TFR2* leads to a more severe iron loading phenotype than loss of either protein alone supports the notion that these proteins could work in parallel to regulate hepcidin expression. Even if HFE and TFR2 act by parallel pathways, cross talk between them may account for observations suggesting their co-dependence. We have postulated that both proteins initiate hepcidin induction through the BMP-HJV signaling pathway [24]. The molecular participants in this signaling pathway include neogenin, a DCC (deleted in colorectal cancer) family member that interacts with HJV [40–42]. Loss of neogenin leads to decreased BMP-mediated hepcidin signaling [43]. Either HFE or TFR2 might independently regulate neogenin function or expression leading to differential hepcidin expression. Additional work will undoubtedly be necessary to better understand the role of HFE and TFR2 in TF-Fe_2 sensing and regulation of liver hepcidin expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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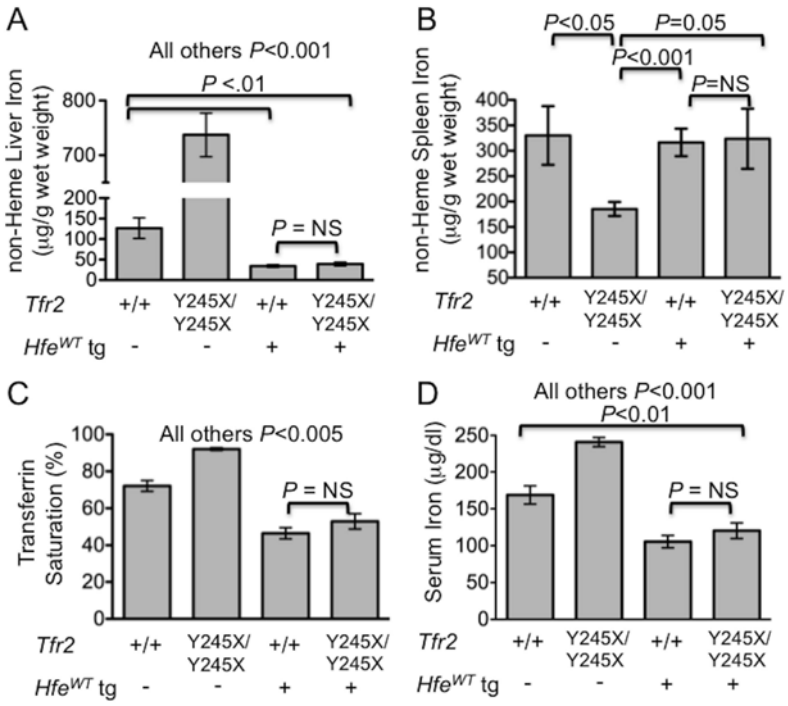

Figure 1. Phenotypic analysis of *Tfr2*$^{Y245X/Y245X}$ mice expressing a wildtype hepatocyte-specific *Hfe* transgene (*Hfe*^{WT}tg)

Analysis of (A) non-heme liver iron and (B) spleen iron (μg/g wet weight), and (C) serum transferrin saturation (%) and (D) serum iron (μg/dl). WT (n=9), *Tfr2*$^{Y245X/Y245X}$ (*n*=17), *Hfe*^{WT} (*n*=11) and *Tfr2*$^{Y245X/Y245X}$ *Hfe*^{WT} (*n*=8) are depicted. Ratios are expressed± SEM. p-values were calculated using Student's t-test.

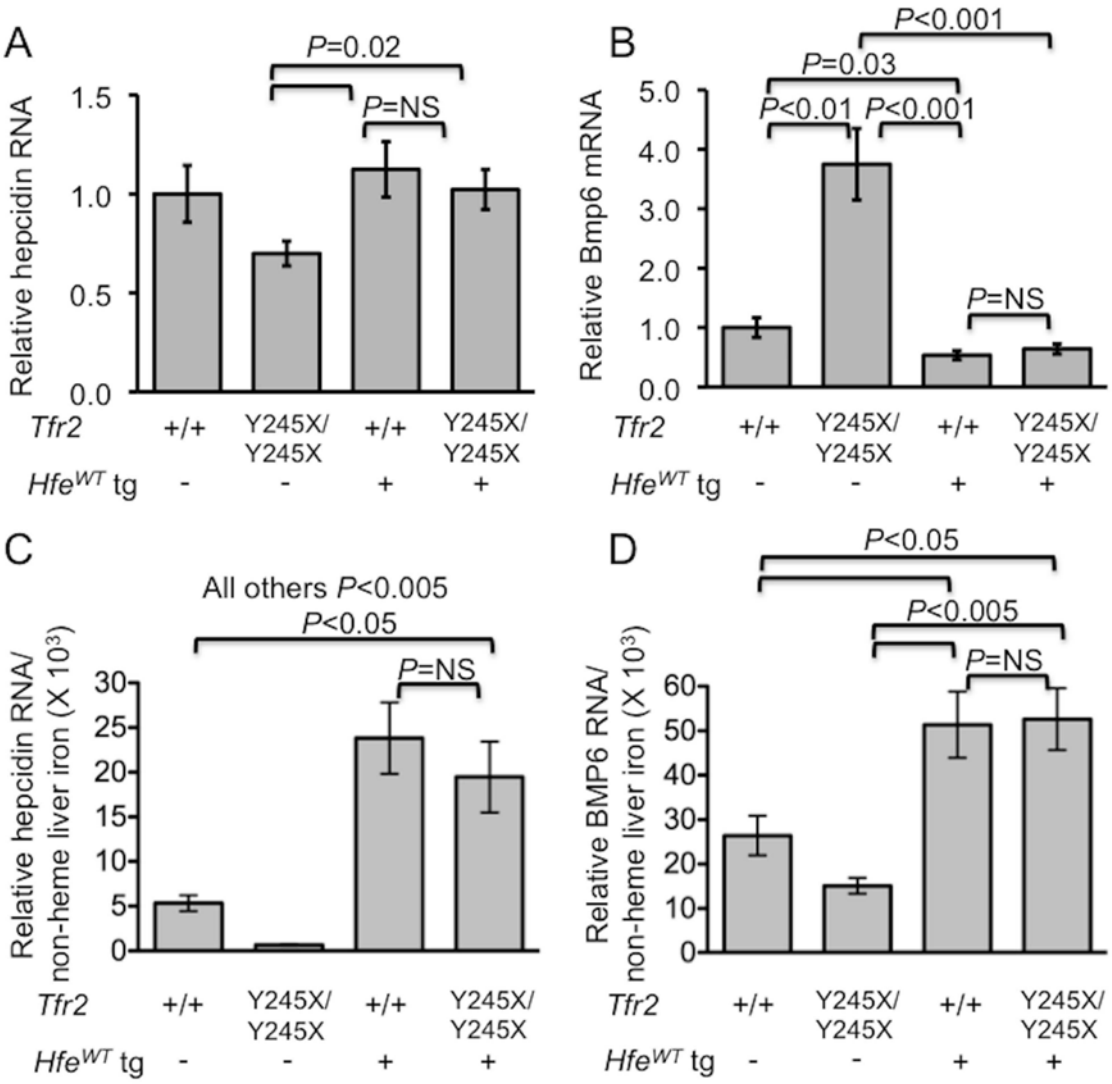

Figure 2. Analysis of BMP signaling in *Tfr2*$^{-/-}$ mice expressing a wildtype hepatocyte-specific *Hfe* transgene (*Hfe*^{WT} tg)

Total mRNA was harvested from wild-type (WT), *Tfr2*$^{Y245X/Y245X}$, *Hfe*^{WT} transgenic or *Tfr2*$^{Y245X/Y245X}$ *Hfe*^{WT} transgenic livers (n=6 for each genotype) and hepcidin (*Hamp*) (A), and *Bmp6* (B) mRNA was assessed by quantitative real-time PCR, normalized to β-actin (*Actb*), and then expressed relative to the WT value whose mean was defined as 1.0. Hepcidin (C) or BMP6 (D) mRNA expression relative to non-heme liver iron. Ratios are expressed ±SEM. p-values were calculated using Student's t-test.

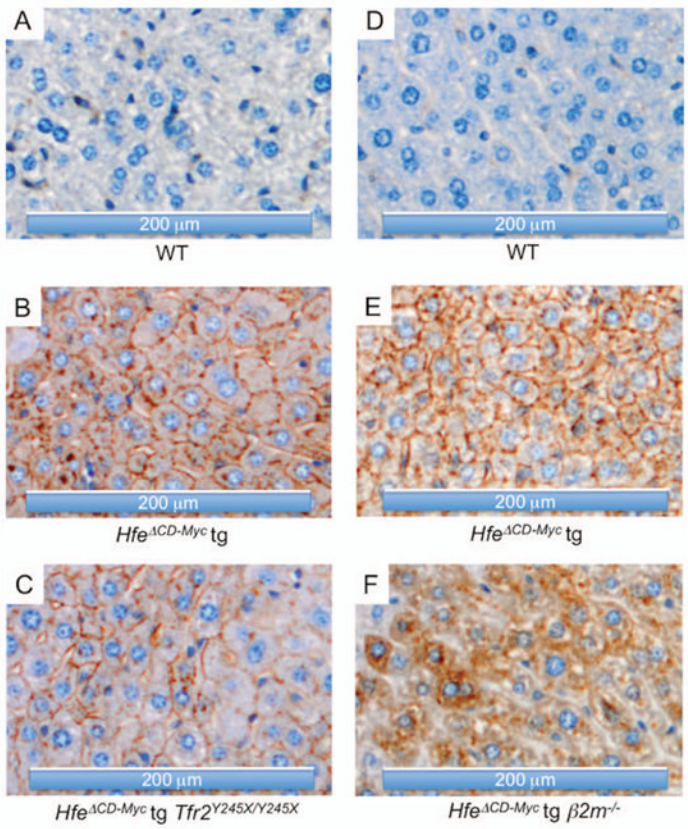

Figure 3. Immunochemical analysis of *Hfe* cMyc transgene expression in $\beta 2m^{-/-}$ or $Tfr2^{Y245X/Y245X}$ mice

Liver immunohistochemistry for cMyc epitope using anti-cMyc antibody (Abcam) in WT (A), $Hfe^{\Delta CD\text{-}MYC}$ (B), $Tfr2^{Y245X/Y245X}$ $Hfe^{\Delta CD\text{-}MYC}$ (C) or WT (D), $Hfe^{\Delta CD\text{-}MYC}$ (E) and $\beta 2m^{-/-}$ $Hfe^{\Delta CD\text{-}MYC}$ animals. Original magnification: 80x.

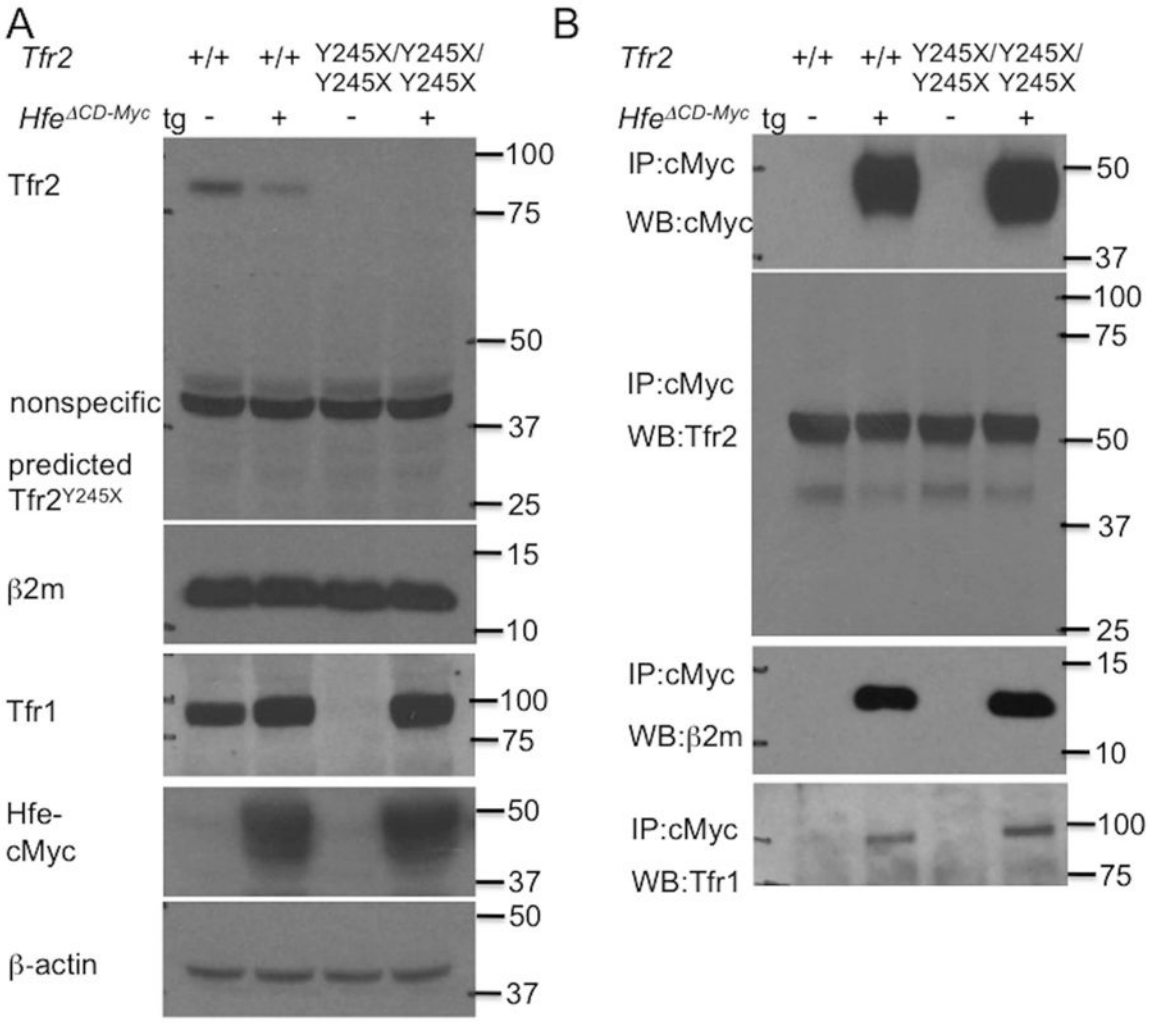

Figure 4. Western blot analysis of *Hfe* cMyc transgene expression and immunoprecipitation in *Tfr2*$^{Y245X/Y245X}$ mice

Liver protein lysates were analyzed for Tfr2 (A, top panel), β2m (A, second panel), Tfr1 (A, third panel) or Hfe-cMyc protein (A, fourth panel) in 8-week-old wild-type (WT), *Tfr2*$^{Y245X/Y245X}$, Hfe-cMyc transgenic (*Hfe*$^{\Delta CD\text{-}MYC}$ tg), *or Tfr2*$^{Y245X/Y245X}$ Hfe-cMyc transgenic (*Hfe*$^{\Delta CD\text{-}MYC}$ tg) animals by Western blot. Equivalent loading of liver lysates was confirmed by immunoblot analysis for β-actin (A, lower panel). The *Hfe*$^{\Delta CD\text{-}MYC}$ protein product was immunoprecipitated from liver lysates (B) using a rabbit anti-cMyc antibody. Hfe$^{\Delta CD\text{-}MYC}$, Tfr2, Tfr1 or β2m were detected on immunoblots using rabbit anti-cMyc, -Tfr2 or -β2m or mouse anti-Tfr1. Immunoprecipitation (IP) antibody and Western blot (WB) antibody as noted.

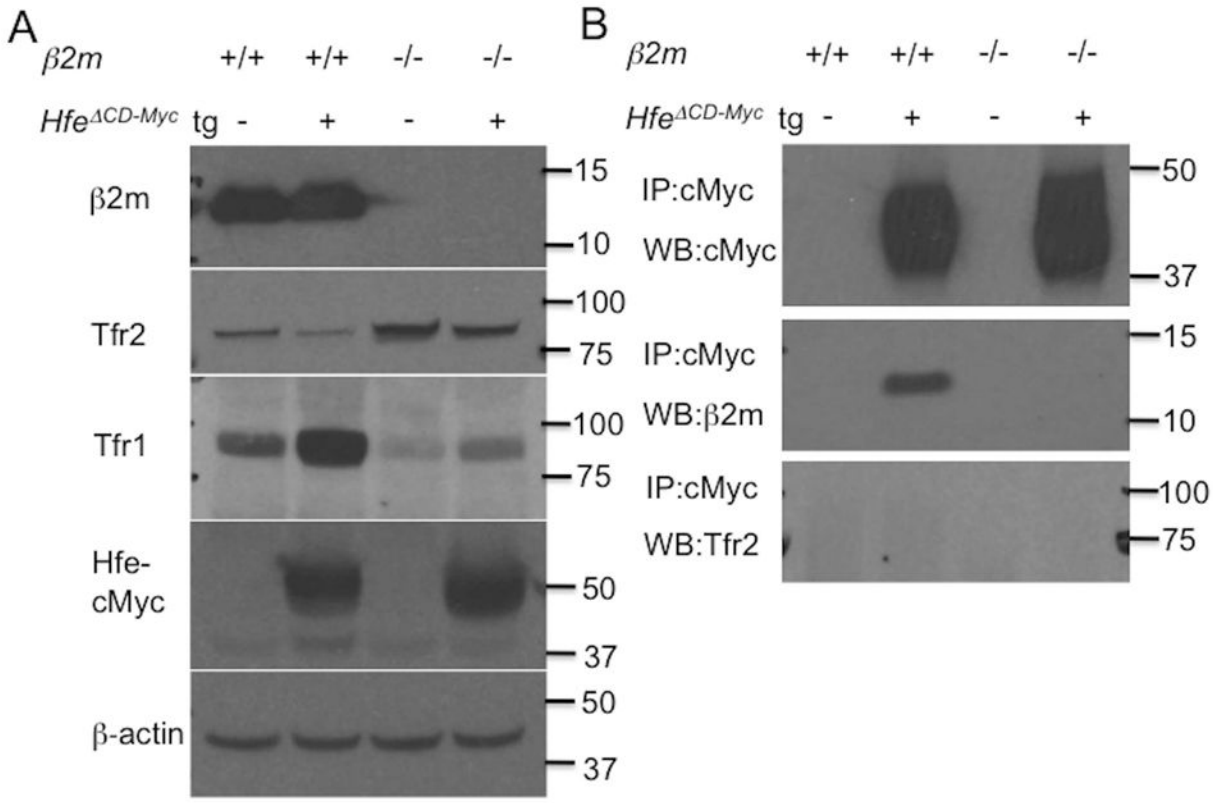

Figure 5. Western blot analysis of *Hfe* cMyc transgene expression and immunoprecipitation in $\beta 2m^{-/-}$ mice

Liver protein lysates were analyzed for β2m protein (A, top panel), Tfr2 (A, second panel), Tfr1 (A, third panel) or Hfe-cMyc protein (A, fourth panel) in 8-week-old wild-type (WT), $\beta 2m^{-/-}$, Hfe-cMyc transgenic ($Hfe^{\Delta CD\text{-}MYC}$ tg), or $\beta 2m^{-/-}$ Hfe-cMyc transgenic ($Hfe^{\Delta CD\text{-}MYC}$ tg) animals by Western blot. Equivalent loading of liver lysates was confirmed by immunoblot analysis for β-actin (A, lower panel). The $Hfe^{\Delta CD\text{-}MYC}$ protein product was immunoprecipitated from liver lysates (B) using a rabbit anti-cMyc antibody and $Hfe^{\Delta CD\text{-}MYC}$, Tfr2 or β2m were detected on immunoblots as in Figure 3. Immunoprecipitation (IP) antibody and Western blot (WB) antibody as noted.

Table I

Hematologic Features of *Tfr2* $^{Y245X/Y245X}$ *Hfe* ^{WT} Transgenic Animals

Genotype	n	Hgb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	RDW (%)	Retic (%)	Chr (pg)
WT	9	14.8±0.2	51.8±0.8	51.2±0.8	14.7±0.2	12.6±0.2	3.2±0.3	15.4±0.3
Tfr2 $^{Y245X/Y245X}$	17	14.9±0.2	51.8±0.6	51.5±0.4	14.9±0.1	14.5±0.3 *	3.3±0.3	16.1±0.1 ¶
Hfe ^{WT} tg	11	13.1±0.2 *†	46.2±0.9 *†	43.5±0.7 *†	12.3±0.3 *†	16.6±0.6 $^{*\Delta}$	3.6±0.2	13.2±0.3 *†
Tfr2 $^{Y245X/Y245X}$ *Hfe* ^{WT} tg	8	14.2±0.3 $^{\#}$	51.4±1.1 ^{f}	46.0±1.5 $^{¶\Delta}$	12.7±0.4 *†	19.7±1.1 $^{*\&\pi}$	3.1±0.7 $^{\&}$	15.1±0.4 ^{f}

The red blood cell parameters hemoglobin (Hgb), hematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH), red cell distribution width (RDW), reticulocyte count (Retic), and reticulocyte mean cell hemoglobin (CHr) were measured in 8-week-old female wild-type (WT), *Tfr2* $^{Y245X/Y245X}$, *Hfe* ^{WT} tg and *Tfr2* $^{Y245X/Y245X}$ *Hfe* ^{WT} tg mice. Data are presented as mean ± SEM. p values were calculated by Student's t test using Microsoft Excel.

* P<0.001,

§ P<0.005 or

¶ P<0.05 versus WT;

† P<0.001,

$^{\Delta}$ P<0.01 or

$^{\&}$ P<0.05 versus *Tfr2* $^{-/-}$;

^{f} P<0.005,

$^{\#}$ P<0.01 or

$^{\pi}$ P<0.05 versus *Hfe* ^{WT} tg.