LIVER DISEASE

First phenotypic description of transferrin receptor 2 knockout mouse, and the role of hepcidin

D F Wallace, L Summerville, P E Lusby, V N Subramaniam

See end of article for authors' affiliations
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Correspondence to:
Dr V N Subramaniam, The Membrane Transport Laboratory, Queensland Institute of Medical Research, 300 Herston Rd, Herston, QLD 4006, Australia; nathanS@qimr.edu.au

Background: Transferrin receptor 2 (TfR2) is a key molecule involved in the regulation of iron homeostasis. Mutations in humans cause type 3 haemochromatosis and a targeted mutation in mice leads to iron overload with a similar phenotype. We have previously described the generation of a complete *TfR2*-knockout (KO) mouse.
Aims: The aims of this study were to determine the phenotype and analyse expression of iron related molecules in the liver, duodenum, and spleen of homozygous *TfR2*-KO, heterozygous, and wild-type mice.
Methods: Serum and tissue iron levels were determined in 10 week old male mice. Expression of iron related mRNA transcripts were analysed in the liver, duodenum, and spleen using real time polymerase chain reaction. Expression of iron related proteins in the liver were analysed by immunoblotting and immunohistochemistry.
Results: Homozygous *TfR2*-KO mice had no TfR2 protein expression and developed significant iron overload typical of *TfR2* associated haemochromatosis. In the liver of *TfR2*-KO mice there was no upregulation of *hepcidin* mRNA or prohepcidin protein in response to iron loading.
Conclusions: Our results suggest that TfR2 is required for iron regulated expression of hepcidin and is involved in a pathway related to Hfe and hemojuvelin.

Transferrin receptor 2 (TfR2) is a homologue of transferrin receptor 1 (TfR1), the primary molecule responsible for the uptake of transferrin bound iron into cells.[1] Unlike the ubiquitously expressed TfR1, TfR2 is thought to be expressed almost exclusively in the liver and erythroid cells.[1] *TfR2* mRNA contains no iron responsive elements and does not appear to be regulated by iron.[2] TfR2 can bind transferrin but at a much reduced affinity compared with TfR1.[3 4] The exact role of TfR2 in iron metabolism is unknown although it is evident that it plays an important function in maintaining iron homeostasis as mutations in the *TfR2* gene lead to haemochromatosis in humans.[5 6] Mice with a targeted mutation of *TfR2* (Y245X, orthologous to human Y250X) develop iron overload with a similar phenotype to patients with *TfR2* associated haemochromatosis.[7]

Hepcidin, originally described as an antimicrobial peptide, has been shown to be a key regulator of iron homeostasis.[8–10] Hepcidin is produced in the liver and is secreted into the circulation in response to inflammation or increased iron stores.[10 11] Hepcidin is a negative regulator of iron absorption and iron release from cells. Mice have two *hepcidin* genes located adjacent to each other on chromosome 7.[10] This differs from humans and rats which only have one gene. Mutations of hepcidin in humans and knockout in mice lead to a severe form of haemochromatosis.[12 13] Regulation of hepcidin appears to be impaired in hereditary haemochromatosis. In patients with *HFE* associated haemochromatosis and in *Hfe* knockout mice, there is loss of regulation of *hepcidin* mRNA expression, causing hepcidin levels to remain low despite increased iron stores.[14 15] Therefore, it appears that HFE is involved in the iron induced pathway of hepcidin expression. One study has suggested that HFE is also involved in the inflammation induced pathway,[16] but this is controversial, and other groups have shown that the inflammation induced pathway is independent of HFE[17 18] and that this pathway is also independent of TfR2.[18] The recently identified molecule mutated in juvenile haemochromatosis, hemojuvelin (Hjv), is also thought to be a key player

in the regulation of hepcidin expression.[19] However, the location of Hjv within the hepcidin regulatory pathway is not yet known.

Previous studies have shown that targeted mutations and knockout of genes in mice do not always produce exactly the same phenotype, as some mutant proteins can retain partial function. For example, mice with a full knockout of the *Hfe* gene develop more severe iron overload than mice with the targeted *Hfe*-C282Y mutation (the primary cause of *HFE* associated haemochromatosis in humans), suggesting that the C282Y mutation may not completely abrogate the function of HFE.[20] Therefore, to complement studies on mice with the targeted *TfR2*-Y245X mutation, we have generated and studied mice with a full knockout of the *TfR2* gene. We used a Cre recombinase:*loxP* strategy to delete exons 2–6 of the mouse *TfR2* gene.[21] These mice have complete absence of TfR2 protein expression, as determined by immunoblotting, with TfR2 antibodies raised against both the amino terminal cytoplasmic domain and the carboxyl terminal extracellular domain. We have used these mice to investigate the molecular basis of iron overload and study expression of iron related molecules in the liver, duodenum, and spleen.

METHODS
Mice
Mice with a deletion of exons 2–6 of the *TfR2* gene were generated using the Cre recombinase:*loxP* system, as previously described.[21] Heterozygous *TfR2*-knockout (KO) mice on a mixed 129/C57BL6/J strain background were intercrossed to produce homozygous, heterozygous, and wild-type mice. Ten or 11 male mice from each group were sacrificed at 10 weeks of age and tissues and blood were taken for further analysis. All experiments were performed using littermates to

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Abbreviations: DcytB, duodenal cytochrome B; DMT1, divalent metal transporter 1; Fpn1, ferroportin; Hjv, hemojuvelin; KO, knockout; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2; PCR, polymerase chain reaction; PBS, phosphate buffered saline

Table 1 Real time polymerase chain reaction primers

Gene	Sequence (5′-3′)	Reference
β-actin	F: GAC GGC CAA GTC ATC ACT ATT G	Dupic[24]
	R: CCA CAG GAT TCC ATA CCC AAG A	
Ferroportin 1	F: TTG CAG GAG TCA TTG CTG CTA	Dupic[24]
	R: TGG AGT TCT GCA CAC CAT TGA T	
Hfe	F: CTG AAA GGG TGG GAC TAC ATG TTC	Dupic[24]
	R: GGA CAC CAC TCC CAA CTT CGT	
TfR1	F: CAT GAG GGA AAT CAA TGA TCG TA	Dupic[24]
	R: GCC CCA GAA GAT ATG TCG GAA	
DMT1	F: GGC TTT CTT ATG AGC ATT GCC TA	Dupic[24]
	R: GGA GCA CCC AGA GCA GCT TA	
DcytB	F: GCA GCG GGC TCG AGT TTA	Dupic[24]
	R: TTC CAG GTC CAT GGC AGT CT	
Hephaestin	F: TTG TCT CAT GAA GAA CAT TAC AGC AC	Dupic[24]
	R: CAT ATG GCA ATC AAA GCA GAA GA	
TfR2	F: CTA TCT GGT CCT GAT CAC CCT	
	R: TCA GGG TTG ACA TCT TCA TCG A	
Total hepcidin	F: AGA GCT GCA GCC TTT GCA C*	
	R: GAG GTC AGG ATG TGG CTC TA	
Hepcidin1	F: AGA GCT GCA GCC TTT GCA C*	
	R: ACA CTG GGA ATT GTT ACA GCA TTT A	
Hepcidin2	F: AGA GCT GCA GCC TTT GCA C*	
	R: GGA GGG TTT GTT ACA GCA CTG	
Hemojuvelin	F: ATG CCG TGT CCA AGG AGC TT	
	R: TCC ACC TCA GCC TGG TAG AC	

*The same forward (F) primer was used for total hepcidin, hepcidin 1, and hepcidin 2.
R, reverse; DMT1, divalent metal transporter 1; DcytB, duodenal cytochrome B; TfR2, transferrin receptor 2.

avoid any strain background effects. All mice were maintained on standard laboratory chow (Norco Stockfeeds, South Lismore, Australia). Animals received humane care and study protocols were approved by the Queensland Institute of Medical Research Animal Ethics Committee.

Antibodies

Anti-TfR1 antibodies were purchased from Zymed Laboratories (San Francisco, California, USA), antiferritin antibodies from the Binding Site (UK), and antiactin antibodies from Sigma-Aldrich (Castle Hill, Australia). Antibodies against mTfR2 were generated in rabbits using a recombinant protein comprising the amino terminal 81 amino acids of the mouse TfR2 cytoplasmic domain. Antiprohepcidin antibodies were generated using recombinant GST-mouse prohepcidin corresponding to amino acids 19–83.[22]

Measurement of iron indices

Transferrin saturation was measured using an iron and iron binding capacity kit (Sigma-Aldrich). Liver iron concentration was measured using the method of Torrance and Bothwell.[23] The Perls' Prussian blue staining method was used for detecting iron in liver sections.

Real time polymerase chain reaction (PCR) quantification of mRNA

RNA was isolated from liver, duodenum, and spleen using the RNeasy mini kit (Qiagen, Clifton Hill, Australia) according to the manufacturer's instructions. Any contaminating genomic DNA was removed by treatment with RQ1 RNase free DNaseI (Promega, Annandale, Australia). RNA (1–2 μg) was reverse transcribed using Superscript III (Invitrogen, Mulgrave, Australia) according to the manufacturer's instructions. Real time reaction mixes contained 2 μl of cDNA (diluted 1:10 or 1:20), 200 nM of each primer (table 1), and SYBR green PCR master mix (Applied Biosystems, Warrington, UK). Reactions were performed on the Rotorgene RG3000 (Corbett Research, Brisbane, Australia) using the following conditions: two minutes denature at 95℃ followed by 50 cycles of 95℃ for 30 seconds, 55℃ for 30 seconds, and 72℃ for 30 seconds. Fluorescence

due to SYBR green binding to double stranded DNA was measured during the extension steps. Melt curve analysis was routinely performed to monitor primer dimer levels by raising the temperature from 50℃ to 99℃ at 1℃/minute while monitoring fluorescence continuously. All experiments were performed in duplicate, and a single batch of cDNA was used for all genes analysed. A standard curve was performed for each run comprising serial dilutions of pooled cDNA to ensure the integrity of the results. Expression of all target genes was determined by normalising to the respective β-actin levels.

Western blotting

Liver homogenates (100 μg) were electrophoresed on either 10% Tris-glycine sodium dodecyl sulphate-polyacrylamide gels or on 4–12% Novex Bis-Tris gradient gels with a MES buffering system (Invitrogen, Mulgrave, Australia), transferred onto Hybond-C+ or PVDF membrane, and blocked in 10% skim milk powder and 0.5% Tween 20 in phosphate buffered saline (PBS) (blocking buffer) overnight at 4℃. Anti-TfR2 (1 μg/ml), anti-TfR1 (1 μg/,ml), antiferritin (1:200), antiprohepcidin (2 μg/ml) or antiactin (1:400) was diluted in blocking buffer and applied to the blots for one hour at room temperature. Blots were washed extensively with 0.1% Tween 20 in PBS and then incubated with antirabbit, antimouse, or antisheep horseradish peroxidase for one hour at room temperature. Blots were washed and Supersignal West Pico chemiluminescent substrate (Pierce Chemical Co., Rockford, Illinois, USA) was applied to the blot and exposed to film. Protein expression was quantified by densitometry and normalised to levels of β-actin.

Immunohistochemistry

Portions of liver were placed in 10% buffered formalin for two hours, transferred into 70% ethanol, processed, and embedded in paraffin. Tissue sections (3 μm) were subjected to citrate buffer antigen retrieval and blocked with 10% goat serum for 30 minutes. Sections were incubated with anti-TfR2 (10 μg/ml) and antiprohepcidin (10 μg/ml) antibodies at 4℃ in the dark overnight. Slides were rinsed with PBS, incubated with biotinylated goat antirabbit antibody (Dako A/S, Denmark) for one hour, rinsed briefly in PBS, and
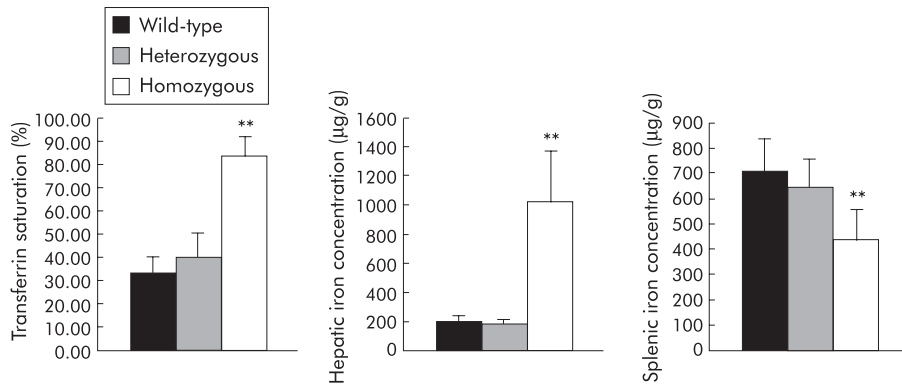
Figure 1 Measurement of iron indices. Mean values for transferrin saturation, hepatic iron concentration, and splenic iron concentration are shown for 10 week old wild-type, heterozygous, and homozygous *transferrin receptor 2*-knockout mice. Error bars denote SDs. **p<0.01.

incubated with StreptABComplex/horseradish peroxidase for 30 minutes. After rinsing with PBS the slides were incubated with Sigma Fast 3,3'-diaminobenzidine tablets in PBS for four minutes. The slides were counterstained with Harris haematoxylin, dehydrated through alcohol series and xylene, and mounted with DePeX mounting medium. Images were taken using an Olympus DPII digital camera.

Statistics

Variables were compared between groups using both parametric (one way ANOVA and the Student's *t* test) and nonparametric (Kruskal-Wallis and Mann-Whitney) statistical tests using the SPSS software package. Correlations were performed between variables using Microsoft Excel. A p value <0.05 was considered statistically significant.

RESULTS

Assessment of iron status

Measurement of iron indices in *TfR2*-KO mice showed that they developed significant iron overload by 10 weeks of age. Transferrin saturation was significantly elevated (83.7 *v* 36.7%; p<0.001) in homozygous *TfR2*-KO mice compared with wild-type and heterozygous mice (fig 1). Hepatic iron concentration was approximately fivefold higher in homozygous *TfR2*-KO mice (1023.6 *v* 188.8 µg/g; p<0.001). There was no significant difference in transferrin saturation or hepatic and splenic iron concentrations between wild-type and heterozygous mice. Homozygous *TfR2*-KO mice had significantly less splenic iron than wild-type and heterozygous mice (fig 1).

The pattern of hepatic iron loading in *TfR2*-KO mice was assessed by Perls' staining of liver sections. Iron accumulation was clearly visible in hepatocytes with a gradient from periportal to pericentral regions (fig 2), a pattern typical of that seen in patients with *TfR2* associated haemochromatosis[25]

and in mice with mutant *TfR2*.[7] No staining for iron was detected in wild-type or heterozygous mice.

Expression of iron related genes in the liver, duodenum, and spleen

Levels of iron related mRNAs were measured in the livers of homozygous *TfR2*-KO, heterozygous, and wild-type mice using real time PCR. *TfR2* mRNA was absent in the liver of homozygous mice and levels in heterozygous mice were approximately half those of wild-type mice (table 2). *TfR1* and *divalent metal transporter 1* (*DMT1*) mRNA levels were significantly reduced (eightfold and 1.5-fold, respectively) in homozygous mice compared with wild-type and heterozygous mice (table 2). Hepcidin 2 mRNA levels were significantly higher in homozygous mice (p = 0.016). However, levels of total hepcidin and hepcidin 1 mRNA were similar in all three groups and there was a close correlation between the two ($r = 0.92$; $p = 2.17 \times 10^{-13}$). Hepcidin 2 mRNA levels did not correlate with either total hepcidin or hepcidin 1 mRNA, suggesting that hepcidin 1 accounts for most of the total hepcidin and is the predominant form produced in the liver. This agrees with previously published data in C57BL/6J mice where hepcidin 1 was found to be the predominant form.[26]

Levels of *Hfe*, *Hjv*, and *ferroportin1* (*Fpn1*) mRNA in the liver were similar in all groups and there was no significant difference when compared using the Student's *t* test. However, the non-parametric Mann-Whitney test suggested there was significantly less *Hfe* and *Hjv* mRNA in the livers of homozygous *TfR2*-KO mice compared with wild-type and heterozygous mice (p = 0.035 and 0.007, respectively). On further inspection we noted that there was one mouse with much higher levels of *Hfe*, *Hjv*, and *Fpn1* in the homozygous group. When this mouse, or outlier, was removed from the analysis the Student's *t* test also delivered a significant result for both *Hfe* and *Hjv* (p<0.001 and p = 0.001, respectively),
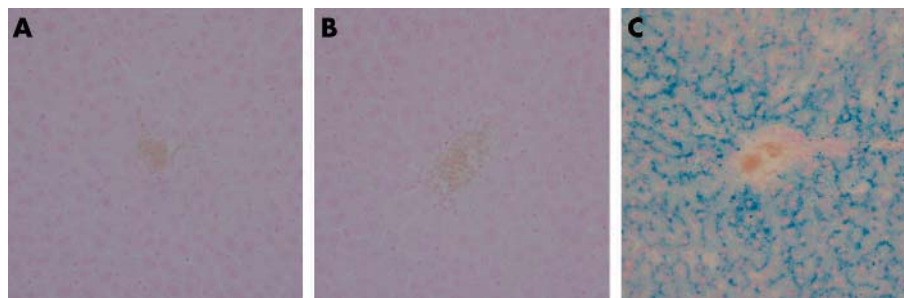
Figure 2 Perls' staining of liver sections. Liver sections from 10 week old wild-type (A), heterozygous (B), and homozygous (C) *transferrin receptor 2*-knockout mice stained with Perls' Prussian blue for iron. Original magnification 20×.

Table 2 Quantification of mRNA transcripts in the liver, duodenum, and spleen

Tissue	mRNA	Wild-type	Heterozygous TfR2-KO	Homozygous TfR2-KO
Liver	TfR2	0.1887 (0.0868)	0.0989* (0.0453)	0.0000** (0.0000)
	TfR1	0.00464 (0.00359)	0.00421 (0.00270)	0.00053** (0.00050)
	Total hepcidin	67.00 (61.09)	81.16 (45.58)	59.06 (41.30)
	Hepcidin1	19.50 (14.55)	20.47 (12.76)	12.90 (10.11)
	Hepcidin2	3.049 (3.542)	4.551 (4.095)	9.634* (6.071)
	Hfe	0.0665 (0.0187)	0.0707 (0.0301)	0.0496 [0.0410**] (0.0290 [0.0103])
	Fpn1	0.0245 (0.0100)	0.0251 (0.0146)	0.0287 [0.0187*] (0.0318 [0.0030])
	Hjv	0.0145 (0.0020)	0.0188 (0.0086)	0.0178 [0.0105**] (0.0234 [0.0026])
	DMT1	0.0580 (0.0396)	0.0580 (0.0252)	0.0384* (0.0134)
Duodenum	Fpn1	0.1060 (0.0304)	0.0831 (0.0501)	0.0972 (0.0512)
	DcytB	0.0827 (0.0636)	0.0942 (0.1134)	0.1541 (0.1610)
	TfR1	0.00339 (0.00203)	0.00324 (0.00200)	0.00318 (0.00167)
	DMT1	0.00393 (0.00340)	0.00386 (0.00469)	0.00427 (0.00219)
	Hephaestin	0.001535 (0.000910)	0.000865 (0.000590)	0.000815 (0.000501)
Spleen	Fpn1	0.0221 (0.0156)	ND	0.0158 (0.0101)
	TfR2	0.00122 (0.00090)	ND	0.00000* (0.00000)
	TfR1	0.00807 (0.00474)	ND	0.00338 (0.00370)
	Total hepcidin	0.00357 (0.00493)	ND	0.00213 (0.00163)
	DMT1	0.00154 (0.00075)	ND	0.00208 (0.00338)

Mean (SD) values are shown, normalised to β-actin mRNA levels.
[Outliers removed from analysis]. ND, not done.
*p<0.05; **p<0.01.
DcytB, duodenal cytochrome B; DMT1, divalent metal transporter 1; Fpn1, ferroportin; Hjv, hemojuvelin; KO, knockout; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

with *Hfe* being 1.7- and *Hjv* being 1.6-fold lower in the homozygous group (table 2). The *Fpn1* level was also reduced 1.3-fold in the homozygous group compared with wild-type and heterozygous mice (table 2) but this was only marginally significant when using the Student's t test (p = 0.043) and not significant when using other statistical tests (one way ANOVA, Kruskal-Wallis, and Mann-Whitney).

Levels of iron related mRNAs in the duodenum were similar in all three groups of mice and there was no significant difference in expression of these molecules (table 2). *TfR2* mRNA in the duodenum was virtually undetectable in all three groups. Levels of iron related mRNAs in the spleen were compared between wild-type and homozygous mice only (table 2). *TfR2* mRNA was undetectable in the spleens of homozygous *TfR2*-KO mice. Wild-type mice had approximately 150-fold less *TfR2* in the spleen compared with the liver. Levels of all other mRNAs in the spleen were similar between the two groups, except that there was less *TfR1* in homozygous mice. This approached significance when compared using the Student's *t* test (p = 0.054) and was significant when using the Mann-Whitney test (p = 0.013).
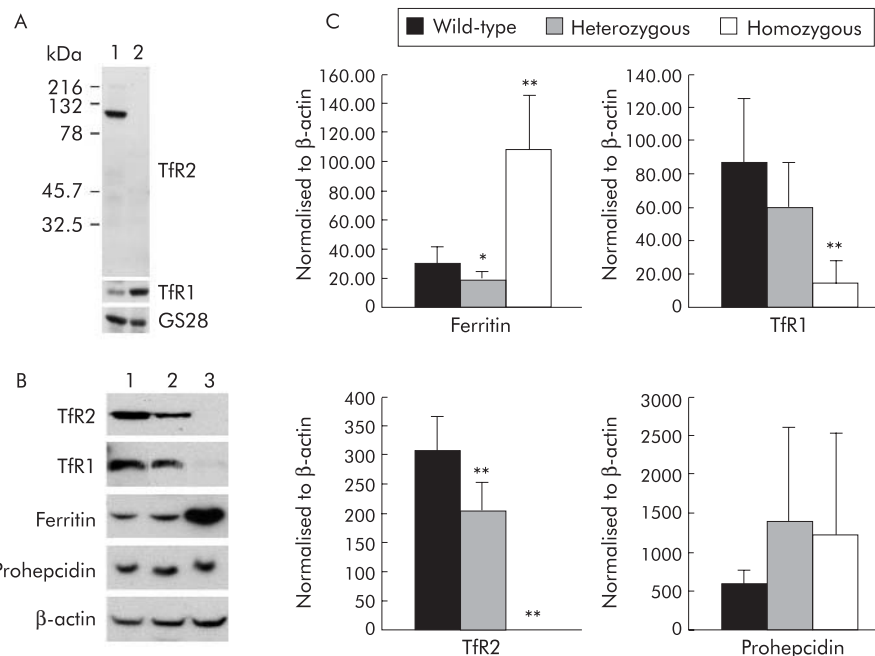
**Figure 3** Quantification of hepatic protein levels. (A) Western blot analysis of 100 μg of mouse liver (lane 1) and kidney (lane 2) homogenates with antibodies against transferrin receptor 2 (TfR2), transferrin receptor 1 (TfR1), and the Golgi marker GS28 as a loading control. Antibodies were specific for TfR2. (B) Total liver protein from 10 week old mice was western blotted with antibodies against TfR2, TfR1, ferritin, prohepcidin, and β-actin. Representative western blot of three mice: lane 1, wild-type; lane 2, heterozygous; lane 3, homozygous *TfR2*-knockout (KO). (C) Quantification of protein levels after normalising to β-actin. Mean (SD) values are shown for wild-type, heterozygous, and homozygous *TfR2*-KO mice. *p<0.05; **p<0.01.
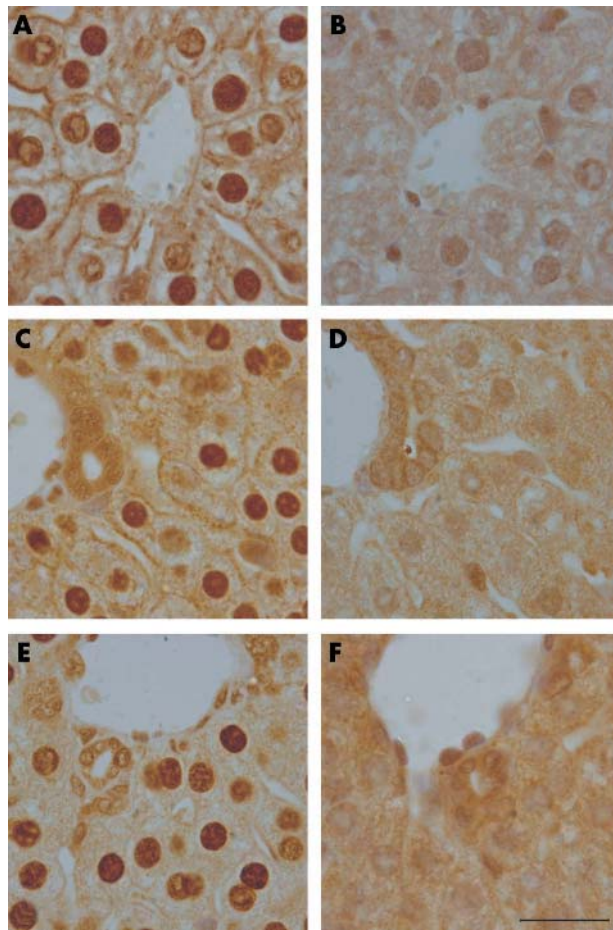
Figure 4 Immunohistochemical localisation of transferrin receptor 2 (TfR2) in the liver. Liver sections from 10 week old wild-type (A,B), heterozygous (C, D), and homozygous (E, F) *TfR2*-knockout mice were incubated with anti-TfR2 antibodies (A, C, E) or control rabbit IgG (B, D, F). Original magnification 100×. Scale bar 200 μm.
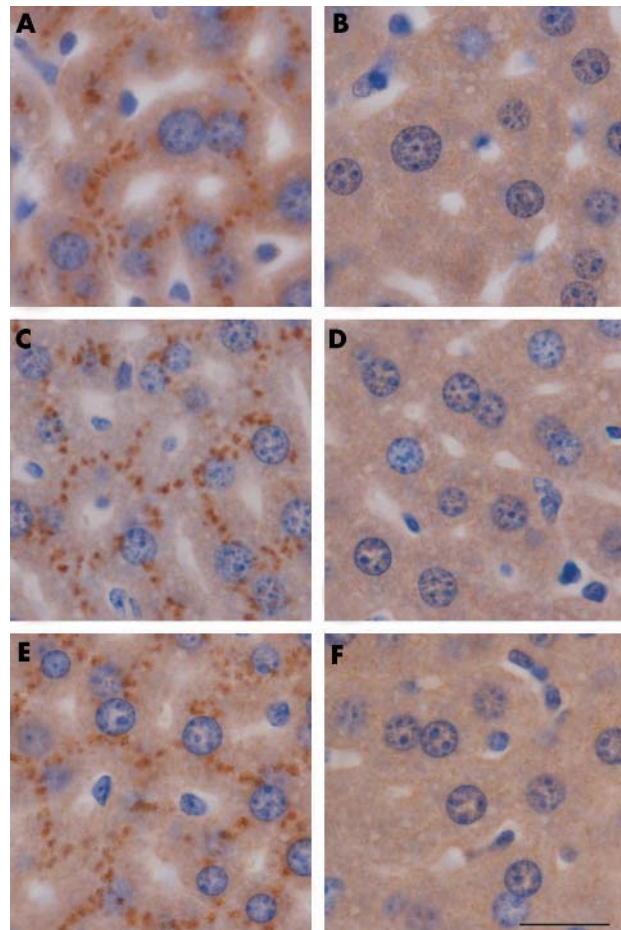
Figure 5 Immunohistochemical localisation of prohepcidin in the liver. Liver sections from 10 week old wild-type (A, B), heterozygous (C, D), and homozygous (E, F) *transferrin receptor 2*-knockout mice were incubated with antiprohepcidin antibodies (A, C, E) or control rabbit IgG (B, D, F). Original magnification 100×. Scale bar 200 μm.

Hepatic protein expression in the liver

Immunoblotting was used to determine expression of key iron related proteins in the liver. Levels of TfR2 protein were determined using a TfR2 specific antibody (fig 3A) and correlated well with levels of *TfR2* mRNA ($r = 0.88$, p<0.001). A representative immunoblot of the key iron related proteins is shown in fig 3B. TfR2 protein was completely absent in the livers of *TfR2*-KO mice. Heterozygous mice had intermediate levels compared with wild-type mice. These results were confirmed using an antibody raised against the extracellular domain of TfR2 and show that any truncated protein lacking exons 2–6 is not produced in knockout mice (data not shown). TfR1 protein levels were significantly decreased (3.9-fold, p<0.001) while hepatic ferritin levels were significantly raised (4.5-fold, p<0.001) in homozygous mice compared with wild-type and heterozygous mice (fig 3), consistent with hepatic iron loading. Levels of hepatic prohepcidin protein varied between animals but there was no significant difference in expression between groups of mice, despite iron loading and increased transferrin saturation in *TfR2*-KO mice. The prohepcidin antibody used detects both mouse prohepcidin 1 and prohepcidin 2 (unpublished observations). Levels of hepatic prohepcidin protein correlated with total *hepcidin* ($r = 0.39$, p = 0.031) and *hepcidin 1* mRNA ($r = 0.42$, p = 0.020) but not *hepcidin 2* mRNA, further confirming that *hepcidin 1* is the predominant form produced in mouse liver.

Immunohistochemistry of TfR2 and prohepcidin in the liver

TfR2 and prohepcidin expression in the livers of homozygous *TfR2*-KO, heterozygous, and wild-type mice was analysed by immunohistochemistry. Three mice from each group were selected for analysis. Staining was similar within each group and typical images are shown in figs 4 and 5. No TfR2 staining was detected in the liver of homozygous *TfR2*-KO mice. In heterozygous and wild-type mice, TfR2 was expressed in hepatocytes and localised to the cell surface, predominantly on the basolateral surface of the plasma membrane (fig 4) with a faint signal observed on the apical surface. Prohepcidin was detected in the livers of all mice consistent with the immunoblotting results. Prohepcidin staining had a punctate ''beaded'' expression pattern reminiscent of the Golgi apparatus and secretory vesicles (fig 5).

DISCUSSION

In this report we have described the phenotype and analysed expression of iron related molecules in the liver, duodenum, and spleen of mice with complete knockout of the *TfR2* gene. These mice develop parenchymal iron overload and features of *TfR2* associated haemochromatosis. There is an increase in transferrin saturation which is accompanied by hepatic iron loading and splenic iron deficiency. In these mice there is no

upregulation of *hepcidin* mRNA or protein expression, as would be expected in mice with high iron stores, and there is slight downregulation of *Hfe* and *Hjv* mRNA.

TfR2-KO mice have an iron overload phenotype which is similar to *TfR2*-Y245X mutant mice and to patients with *TfR2* associated haemochromatosis. Homozygous *TfR2*-KO mice have increased transferrin saturation compared with both wild-type and heterozygous mice. At 10 weeks of age, homozygous mice have approximately fivefold more iron in the liver compared with wild-type and heterozygous mice. This is reflected in an approximate fivefold increase in hepatic ferritin levels. The spleens of *TfR2*-KO mice are relatively iron deficient. Splenic iron deficiency has also been observed in *TfR2*-Y245X mice[7] and *Hfe*-KO mice, and is consistent with the iron deficiency of the macrophage compartment in patients with hereditary haemochromatosis.

The phenotypes of *TfR2* and *HFE* associated haemochromatosis are very similar,[25] suggesting that both TfR2 and HFE either function in the same pathway or in parallel pathways regulating iron homeostasis. It now seems apparent that the final regulatory target of these molecules is the liver expressed peptide hepcidin. In *HFE* associated haemochromatosis and *Hfe*-KO mice there is no upregulation of hepcidin despite increased iron stores.[14][15] While this manuscript was in preparation, two studies have also implicated TfR2 in the iron induced hepcidin pathway.[27][28] Urinary hepcidin levels were low in patients with *TfR2* associated haemochromatosis and *hepcidin* mRNA was not upregulated in mice homozygous for the *TfR2*-Y245X mutation. Hepcidin functions by inhibiting iron release from cells. A recent study has shown that hepcidin achieves this by binding to the iron exporter ferroportin 1 on the cell surface and inducing its internalisation.[29]

In order to examine the effect of complete absence of TfR2, we studied expression levels of iron related molecules in the liver, duodenum, and spleen. While *TfR2*-KO mice had no expression of *TfR2* mRNA or protein in the liver, heterozygous mice had intermediate levels of both *TfR2* mRNA and protein. Low *TfR1* and *DMT1* mRNA levels together with increased levels of ferritin all point to reduced IRP binding activity and iron overload within hepatocytes. We did not see an increase in *Fpn1* mRNA in *TfR2*-KO mice and in fact there was a slight decrease when an outlier was removed from the analysis. This may indicate transcriptional downregulation of *Fpn1* in the livers of *TfR2*-KO mice. The level of Fpn1 protein was not measured and therefore the effect of iron overload on IRE-IRP mediated regulation of Fpn1 protein could not be assessed.

We have shown that expression of *hepcidin* mRNA in the liver is not consistent with the observed hepatic iron loading in *TfR2*-KO mice. Total *hepcidin* and *hepcidin 1* mRNA levels were not increased in homozygous *TfR2*-KO mice despite iron loading. This is consistent with the observed loss of *hepcidin* mRNA regulation in mice with the *TfR2*-Y245X mutation.[28] We did see a significant increase in *hepcidin 2* mRNA in homozygous *TfR2*-KO mice, suggesting that there may be some intact regulation specifically of *hepcidin 2* in response to iron loading. However, levels of *hepcidin 2* in the liver are much lower than *hepcidin 1* and there was no effect of this increase on total *hepcidin* mRNA or prohepcidin protein. Humans only have one *hepcidin* gene and therefore the significance of this finding in the human situation is not clear. Also, another study concluded that hepcidin 2 in mice was not functional in iron metabolism and the effect of hepcidin on iron metabolism was mediated only by hepcidin 1.[30] In addition to *hepcidin* mRNA, we studied expression of prohepcidin protein by immunoblotting. Levels of prohepcidin protein did not increase in *TfR2*-KO mice (fig 3). This is the first analysis of prohepcidin protein levels in mice with defective TfR2 expression.

In addition to hepcidin mRNA and protein levels, we analysed expression of other iron related mRNAs in the liver. On closer inspection of *Hfe* and *Hjv* mRNA expression, a small but statistically significant reduction in these molecules in *TfR2*-KO mice was detected. This difference was observed using non-parametric tests and parametric tests after removing an outlier from the analysis. It is not clear at present whether this reduction in expression is due to iron loading of the liver or a direct consequence of the loss of TfR2. Whether the decrease in *Hfe* and *Hjv* in *TfR2*-KO mice is related to the loss of hepcidin regulation is also not clear. However, it appears that TfR2, Hfe, and Hjv are all related in the iron regulated pathway leading to hepcidin expression.

There was very little difference in expression of iron related mRNAs in the duodenum and spleen of *TfR2*-KO mice. *TfR2* mRNA was undetectable in the duodenum of all mice. In the duodenum, mRNAs encoding the iron transporters *DMT1* and *Fpn1* and facilitators *DcytB* and *hephaestin* were not down-regulated despite iron loading. Contrary to this study, Kawabata *et al* observed an increase in duodenal *DMT1* mRNA levels in *TfR2*-Y245X mice, suggesting that duodenal iron absorption was increased.[28] This difference may be due to the age of the mice studied (four months *v* 10 weeks). Also, Kawabata *et al* specifically measured levels of the exon 1A form of *DMT1* whereas in this study total *DMT1* mRNA was measured. An increase in duodenal iron transporters would not necessarily be expected in *TfR2*-KO mice as the loss of hepcidin regulation in the liver of homozygous *TfR2*-KO mice leads to hepcidin levels comparable with wild-type mice. Even though there was no observed increase in duodenal iron transporters, iron absorption would still be increased relative to iron stores. In the spleen, levels of *TfR2* were approximately 150-fold lower. These results confirm that the primary location of TfR2 is the liver. In the spleen, we observed a reduction in *TfR1* mRNA in homozygous *TfR2*-KO mice. This is contrary to what might be expected; low iron levels in the cell would normally cause stabilisation of *TfR1* mRNA by IRE-IRP mediated mechanisms. Therefore, rather than the iron status of the cell determining expression of TfR1 in the spleen, the low splenic iron concentration in *TfR2*-KO mice may be a consequence of downregulation of TfR1. Mechanisms leading to downregulation of TfR1 in the spleen of these mice are not clear and require further study.

Using immunohistochemistry, we confirmed that TfR2 is absent from the liver of *TfR2*-KO mice. TfR2 localises mainly to the cell surface of hepatocytes, predominantly at the basolateral surface in both wild-type and heterozygous mice, with some staining also detected on the apical surface. The distribution of TfR2 at the plasma membrane of the hepatocyte is consistent with its proposed function. TfR2 could be acting as a sampler of transferrin, perhaps sensing either the iron saturation of transferrin or the amount of diferric transferrin in blood. Activation of TfR2 by transferrin could then cause a signalling cascade resulting in induction of *hepcidin* mRNA expression.

The immunohistochemical localisation of prohepcidin in the liver was the same in homozygous *TfR2*-KO, hetero-zygous, and wild-type mice. Prohepcidin has a ''beaded'' expression pattern which is reminiscent of the Golgi and secretory pathway that we have previously observed in other mouse strains.[22] Therefore, in *TfR2*-KO mice there appears to be no upregulation of hepcidin in response to iron loading and no post-transcriptional regulation of hepcidin protein.

In conclusion, we have shown that mice with complete absence of TfR2 develop haemochromatosis. TfR2 is required for the iron regulated expression of hepcidin. The pathway in which TfR2 functions is likely to involve Hfe and Hjv. While our results cannot definitively place these proteins in precise positions within a pathway, they suggest that TfR2 is

upstream of both Hfe and Hjv in a signalling cascade which results in induction of hepcidin expression. These results support the concept of the liver as the central regulator of body iron homeostasis.

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Authors' affiliations
D F Wallace, L Summerville, P E Lusby, V N Subramaniam, The Membrane Transport Laboratory, Cancer and Cell Biology Division, The Queensland Institute of Medical Research, Brisbane, Queensland, Australia

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