A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression

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Summary

Hereditary hemochromatosis, characterized by iron overload in multiple organs, is one of the most common genetic disorders among Caucasians. Hepcidin, which is synthesized in the liver, plays important roles in iron overload syndromes. Here, we show that a Cre-loxP-mediated liver-specific disruption of SMAD4 results in markedly decreased hepcidin expression and accumulation of iron in many organs, which is most pronounced in liver, kidney, and pancreas. Transcript levels of genes involved in intestinal iron absorption, including Dcytb, DMT1, and ferroportin, are significantly elevated in the absence of hepcidin. We demonstrate that ectopic overexpression of SMAD4 activates the hepcidin promoter and is associated with epigenetic modification of histone H3 to a transcriptionally active form. Moreover, transcriptional activation of hepcidin is abrogated in SMAD4-deficient hepatocytes in response to iron overload, TGF-β, BMP, or IL-6. Our study uncovers a novel role of TGF-β/SMAD4 in regulating hepcidin expression and thus intestinal iron transport and iron homeostasis.

Introduction

Iron is a key component of oxygen-transporting storage molecules, such as hemoglobin and myoglobin. Iron deficiency results in anemia, while iron overload leads to tissue damage and fibrosis. Iron overload in multiple organs/tissues is characteristic of hereditary hemochromatosis, one of the most common genetic disorders among Caucasians. The majority of patients with hereditary hemochromatosis are homozygous for a unique missense mutation (C282Y) that alters a major histocompatibility complex class I-like protein (HFE). Recent investigations have also revealed a number of forms of nonHFE hereditary hemochromatosis that are caused by mutations of several other genes, including ferroportin 1(FPN1) (Montosi et al., 2001; Njajou et al., 2001), transferrin receptor 2 (*TFR2*) (Camaschella et al., 2000), hemojuvelin (*HFE2*) (Papanikolaou et al., 2004), and hepcidin (*HAMP*) (Roetto et al., 2003).

Recent studies indicated that hepcidin (hepcidin-1 in mouse) plays an essential role in regulating iron absorption (Kaplan, 2002; Leong and Lonnerdal, 2004). Hepcidin was independently isolated as a circulating antimicrobial peptide from human urine (Park et al., 2001) and serum (Krause et al., 2000). A lack of hepcidin expression has been associated with iron overload while overexpression of hepcidin results in iron-deficiency anemia in mice (Nicolas et al., 2001, 2002). Furthermore, homozygous mutations of hepcidin in humans have been found to cause severe juvenile hemochromatosis (Roetto et al., 2003). Prohepcidin is produced predominantly by liver, although a number of other organs, such as lung and heart, also express it at much lower levels (Leong and Lonnerdal, 2004). Once cleaved, the mature form, a 25 aa peptide, is secreted into the circulation. Hepcidin in plasma negatively regulates iron absorption in duodenal crypt cells and/or villous enterocytes and inhibits iron release from macrophages (Leong and Lonnerdal, 2004). In HFE hemochromatosis, production of hepcidin appears to be abnormally low (Bridle et al., 2003; Gehrke et al., 2003), suggesting that HFE positively regulates hepcidin expression. Additional factors/conditions, including IL-6, c/EBPα, iron, hypoxia, and inflammation, also regulate hepcidin expression (Courselaud et al., 2002; Nemeth et al., 2004a).

Members of the transforming growth factor β (TGF-β) superfamily play numerous important functions in diverse developmental processes by regulating proliferation, differentiation, and apoptosis (reviewed in Derynck et al., 2001; Pollard, 2001; Wakefield et al., 2001). After activating their transmembrane receptors, TGF-β signaling is transduced into the nucleus by SMADs, a family of at least eight members, of which SMAD4 serves as a central mediator (reviewed in Heldin et al., 1997; Massague, 1998). *SMAD4* is a well-known tumor suppressor gene, and SMAD4 mutations are frequently detected in pancreatic cancer, colon cancer, and gastric polyposis and adenocarcinomas (Friedl et al., 1999; Hahn et al., 1996; Howe et al., 1998; Maesawa et al., 1997; Nagatake et al., 1996; Schutte et al., 1996). Loss of SMAD4 results in lethality at embryonic (E) days 6–7 due to impaired extraembryonic membrane formation and decreased epiblast proliferation (Sirard et al., 1998; Weinstein et al., 1998). Due to the potential functions of SMAD4 at postnatal stages of mammalian development, we have been using the Cre-loxP system to overcome the early lethality and have studied its functions in brain (Zhou et al., 2003) and mammary gland

development and neoplasia (Li et al., 2003). In an attempt to assess the role of SMAD4 in liver development and maintenance, using hepatocyte-specific promoter-driven Cre, we unexpectedly found that the absence of SMAD4 in mouse liver results in iron overload in multiple organs and premature death. We demonstrate that loss of hepatic SMAD4 is associated with dramatically decreased expression of hepcidin in liver and increased duodenal expression of genes involved in intestinal iron absorption, including Dcytb (an apical iron reductase), DMT1 (an apical iron transporter), and ferroportin (a basolateral iron exporter). Furthermore, SMAD4 deficiency also completely blocked hepcidin induction by IL-6 treatment and iron overload.

Results

SMAD4 does not play an indispensable function during postnatal liver development

To obtain liver-specific knockout of *Smad4*, we generated mutant mice carrying a *Smad4* conditional allele (Yang et al., 2002) and an albumin-Cre transgene (Yakar et al., 1999) (*Smad4$^{Co/Co}$;Alb-Cre*). The albumin promoter is active specifically in liver at low levels at E19 and gradually reaches adult levels at 1–2 weeks postnatally (Yakar et al., 1999). Using the Rosa-26 reporter mouse (Soriano, 1999), we detected Cre-mediated recombination in about 40% of hepatic cells at postnatal day 15 (P15) (Figure 1A), which increased to about 90% of cells at P37 (Figure 1B). PCR analysis on DNA isolated from multiple organs, including liver, pancreas, spleen, lung, heart, kidney, and testis, revealed recombination only in liver (Figure 1C). Consistently, Northern blot analysis revealed about 90% reduction of *Smad4* mRNA isolated from *Smad4$^{Co/Co}$;Alb-Cre* liver compared with that of control liver (Figure 1D). These observations indicated that albumin-Cre efficiently disrupts *Smad4* expression in the majority of liver cells.

Next, we analyzed *Smad4$^{Co/Co}$;Alb-Cre* mice for a possible impact on liver development. Histopathologic analysis revealed no obvious defects in mutant liver before 8 months of age (data not shown). This observation indicates that the absence of SMAD4 does not play a major role in liver development. After 8 months, some livers showed fibrosis and accumulation of neutrophils (Figure 1F) and macrophages (Figure 1H), which were not observed in wild-type control livers (Figures 1E and 1G). These histologic lesions might be associated with compromised liver function as evidenced by elevations of SGOT (serum glutamic oxaloacetic transaminase), SGPT (serum glutamate pyruvate transaminase), and bilirubin in older mutant mice (Table S1 in the Supplemental Data available with this article online).

Liver-specific knockout of *Smad4* results in iron overload in multiple organs/tissues

However, *Smad4$^{Co/Co}$;Alb-Cre* mice gradually lost weight with over half of them becoming sick in appearance (rough-looking fur, skinny, loss of muscle mass, and slower movement) at 10 months of age. Some of them (5/18) died at this stage of development. To investigate the possible causes for the wasting of these animals, we performed autopsies on these sick animals and found that they all developed a dark-red pancreas and brownish pigment deposition in multiple organs, including the liver and the proximal tubular epithelium of the kidney (data not shown).

After extensive analysis, we detected a significant overload of iron in samples from mutant mice. Prussian blue staining showed that beginning from the age of 2 months, iron accumulated in all the organs with pigmentation, such as liver (Figures 2A and 2B), pancreas (Figures 2C and 2D), and proximal tubule of the kidney (Figures 2E and 2F). In contrast, mutant bone marrow and spleen demonstrated reduced staining (Figures 2G and 2H, and data not shown), primarily due to the lower iron levels of macrophages in the mutant mice. Quantitative measurement of iron concentrations confirmed alteration of iron levels in these organs (Table 1). Increased serum transferrin saturation levels (about 2-fold, i.e., 78% in mutants vs 42% in controls) were found in all 4 month-old mutant mice examined (n=3). Hepatic iron overload associated with macrophage iron depletion is characteristic of mouse models for human hemochromatosis (Fleming et al., 2002; Nicolas et al., 2001; Santos et al., 1996; Zhou et al., 1998).

Because *Alb-Cre* expresses specifically in liver (Figure 1C) (Yakar et al., 1999), the liver should theoretically be the only affected organ. Thus, it was initially surprising that a liver-specific knockout of SMAD4 resulted in iron overload in multiple organs/tissues. An important feature of liver is that it functions as a secretory organ and is crucial for producing a majority of circulating plasma proteins, which function in many other organs. We therefore hypothesized that the targeted disruption of *Smad4* in the liver must affect normal production of some of these molecules that are responsible for increased iron absorption, leading to a condition mimicking human hemochromatosis.

Dramatically reduced expression of hepcidin in the liver of *Smad4$^{Co/Co}$;Alb-Cre* mice

To test this hypothesis, we used liver RNA and performed a candidate approach to study expression of a number of genes that are involved in iron metabolism, including *Hfe, hepcidin, Trf-1, Trf-2* and *Fpn*. Our RT-PCR (Figure S1), and real-time PCR (Figure 3A) analyses revealed that hepcidin levels decreased about 100-fold in the liver of *Smad4 $^{Co/Co}$;Alb-Cre* mice at 2 to 6 months of age, while expression of other genes was not significantly altered (Figure S1).

Hepcidin represses intestinal iron absorption and enhances macrophage iron sequestration (Ganz, 2005; Viatte et al., 2005). The dramatic decrease in hepcidin in liver tissues from *Smad4$^{Co/Co}$;Alb-Cre* mice led us to evaluate potential downstream targets of hepcidin, including DMT1, FPN1 and DCYTB1 (Frazer et al., 2002; Millard et al., 2004; Nemeth et al., 2004b; Viatte et al., 2005; Yeh et al., 2004). Our real-time PCR analysis revealed that *Dmt1* and *Dcytb1* were each elevated about 3-fold, while *Fpn1* was increased about 2-fold in duodenum, though there were no changes in liver expression of these transcripts (Figure 3B). Increased protein levels in mutant duodenum compared with control were detected by immunohistochemical staining using antibodies against DCYTB1 (Figures 3C and 3D), DMT1 (Figures 3E and 3F), and FPN (Figures 3G and 3H).

TGF-β and BMP positively regulate hepcidin expression through SMAD4

Our data thus far suggested that TGF-β /SMAD4 signals positively regulate hepcidin expression. To test this, we treated wild-type and SMAD4 null (−/−) hepatocyte cell lines with either TGF-β1 or BMP-4. While hepcidin was induced up to 4- to 8-fold

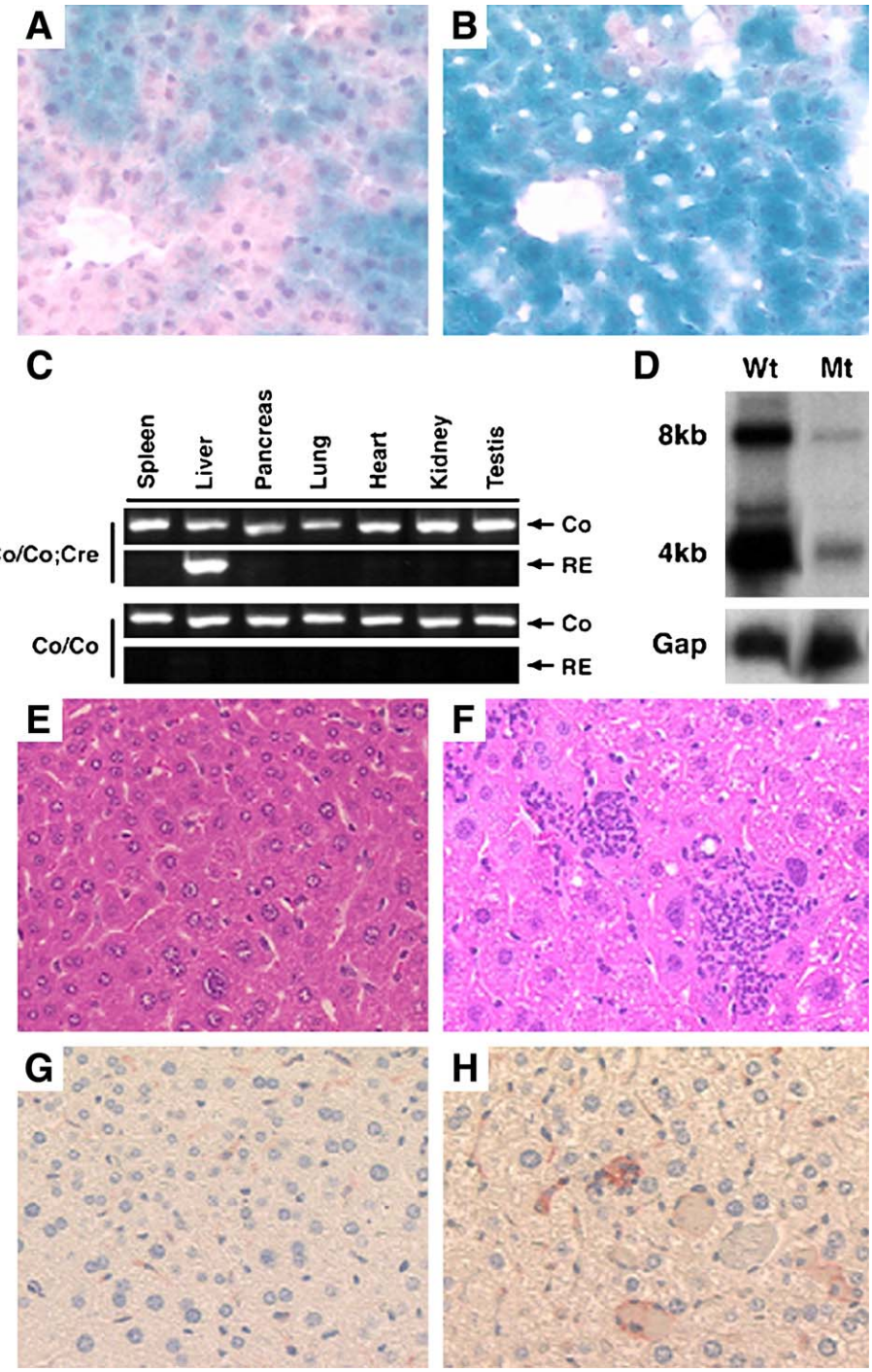

Figure 1. Abnormality of *Smad4*$^{Co/Co}$;*Alb-Cre* mice

A and B) Expression pattern of Alb-Cre in P15 (**A**) and P37 (**B**) mice as assayed by a Rosa-26 reporter. Liver sections are stained with β-gal.

C) *Alb-Cre* promotes recombination of *Smad4* conditional alleles in a liver-specific fashion.

D) Northern blot to show the reduction of both 4 kb and 8 kb *Smad4* transcripts in *Smad4*$^{Co/Co}$;*Alb-Cre* liver.

E–H) Neutrophil invasion (**F**) and accumulation of macrophages (**H**) were observed in the liver of 8-month-old *Smad4*$^{Co/Co}$;*Alb-Cre* mice but not in wild-type liver (**E** and **G**).

with TGF-β1 or BMP-4 in wild-type cells, SMAD4$^{-/-}$ cells had no response (Figures 4A and 4B). This observation indicates that TGF-β1 and BMP-4 require SMAD4 for induction of hepcidin expression. To determine whether the induction by TGF-β1 and BMP-4 in hepcidin expression is direct or indirect, we included cycloheximide in the experiment to inhibit new protein synthesis. Because SMAD4 is not stable with a half-life of about 8 hr, we followed hepcidin expression and SMAD4 levels up to 8 hr after the treatment. Our data indicated that both the TGF-β1 and BMP-4 treatment increased hepcidin transcripts during the first 6 hr, reaching 9- and 6-fold, respectively, and hepcidin expression levels declined at 8 hr after the treatment (Figure 4C). These observations suggest that both TGF-β1 and BMP-4 increase hepcidin expression by directly activating SMAD4 protein, which is consistent with the direct activation of SMAD4 by TGF-β signaling demonstrated previously (reviewed by Heldin et al., 1997; Massague, 1998; ten Dijke and Hill, 2004).

To confirm that the lack of response to TGF-β and BMP observed in SMAD4 null cells is indeed due to the absence of SMAD4, we transfected a *Smad4* expression plasmid into SMAD4 null cells and determined endogenous hepcidin expression. Our data indicated that hepcidin transcription levels were

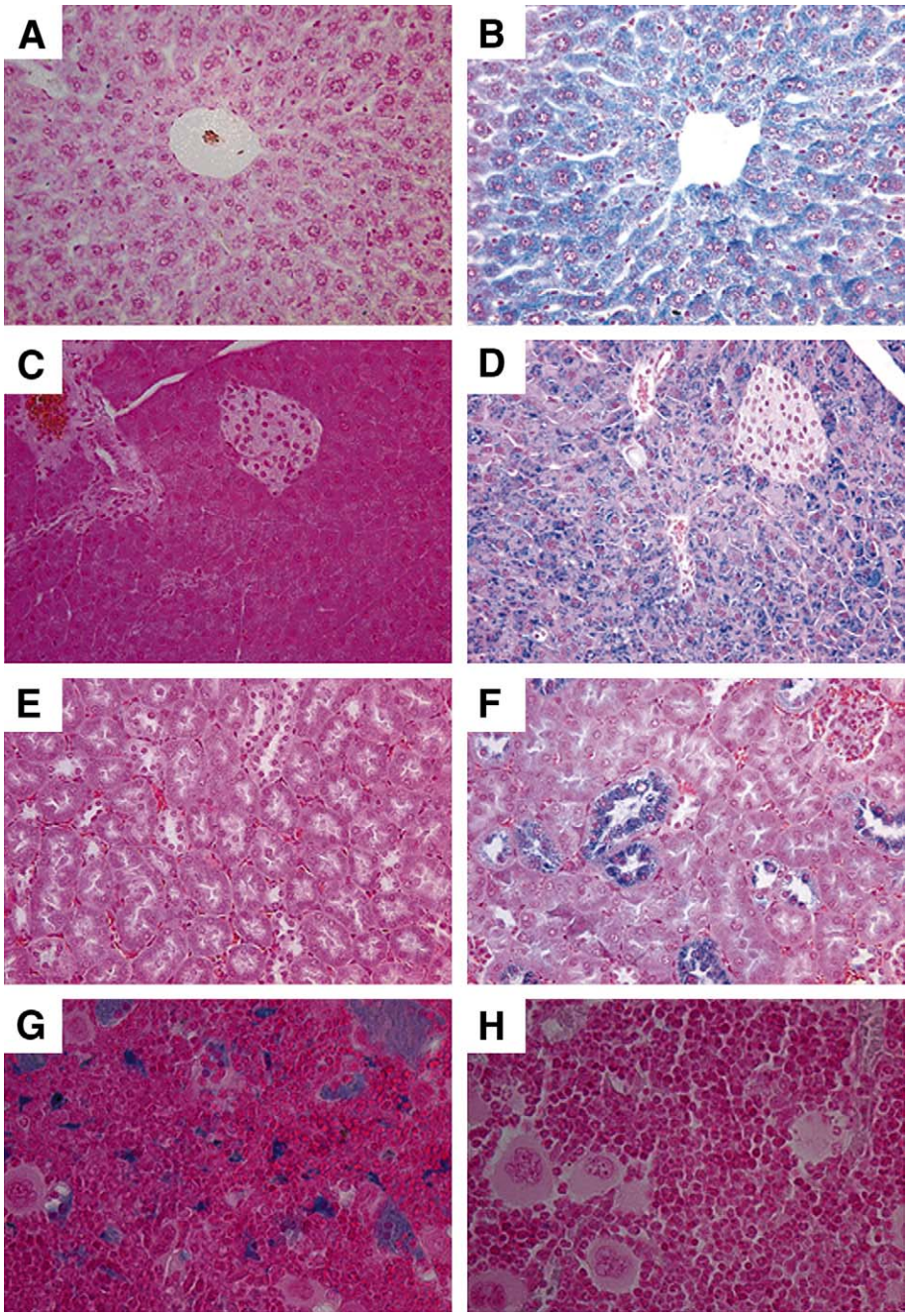

Figure 2. Iron accumulation in multiple organs of 4-month-old *Smad4*$^{Co/Co}$;*Alb-Cre* mice

Prussian blue staining shows iron accumulation in mutant liver (**B**), pancreas (**D**), kidney (**F**), but not in bone marrow (**H**). Iron accumulation did not occur in organs of control littermate mice (**A**, **C**, **E**, and **G**).

induced up to 5-fold when 0.1-1 μg of *Smad4* expression plasmid was transfected (Figure 4D).

SMAD4 positively regulates hepcidin promoter activity

To study the underlying mechanism by which TGF-β and BMP-4 positively regulate hepcidin expression via SMAD4, we performed luciferase reporter assays using a luciferase reporter construct with a fragment of the mouse hepcidin promoter (Courselaud et al., 2002). Wild-type hepatocytes transfected with the luciferase reporter construct had a 2-fold increase in luciferase activity relative to hepatocytes transfected with vector (pGL3B) only. Treatment with BMP4 led to a 3-fold increase in luciferase activity in the reporter construct transfected cells relative to the untreated cells (Figure 4E). A 6-fold increase in luciferase activity was observed when the comparison was made between the reporter construct transfected cells and the vector transfected cells (vector only or vector + BMP-4 treatment, Figure 4E). The increased luciferase activity was likely SMAD4 dependent, as no such increase was observed in SMAD4$^{-/-}$ hepatocytes upon BMP-4 treatment (Figure 4E). To confirm this, we reconstituted SMAD4 in the SMAD4$^{-/-}$ cells by transfecting a *Smad4* expression construct. SMAD4$^{-/-}$ hepatocytes that were cotransfected with the hepcidin promoter-containing luciferase reporter construct and the SMAD4 expression construct showed increasing luciferase activity with increasing amounts of transfected SMAD4 expression construct in a dosage-dependent manner (Figure 4F). We also cotransfected the hepcidin promoter-containing luciferase reporter construct

Table 1. Iron concentrations (μM/g±SD) in *Smad4$^{Co/Co}$;Alb-Cre* mutant and control mice

Samples	Liver	Pancreas	Kidney	Spleen
Control (2 M)	4.73 ± 1.60	3.28 ± 1.63	2.48 ± 1.0	15.51 ± 0.51
Mutant (2 M)	42.99 ± 3.6	35.61 ± 6.29	21.16 ± 11.5	4.62 ± 1.56
Control (4 M)	8.08 ± 1.12	5.99 ± 2.94	4.53 ± 2.72	26.38 ± 5.35
Mutant (4 M)	45.51 ± 2.9	45.78 ± 5.8	30.88 ± 10.9	26.42 ± 14.0
Control (6 M)	4.45 ± 1.7	3.31 ± 0.60	4.475 ± 1.5	17.24 ± 7.8
Mutant (6 M)	41.76 ± 3.5	44.64 ± 4.8	22.36 ± 5.9	11.86 ± 3.3

and the *Smad4* expression construct into HEPA1-6 cells, a cell line that was derived from a SMAD4 wild-type hepatocyte carcinoma (Monga et al., 2002), and found similar SMAD4-dependent increases in luciferase activity (Figure 4F). We conclude that SMAD4 is required to transduce signaling of both BMP and TGF-β subfamilies on the hepcidin promoter.

Histone modification plays an important role in controlling gene expression (He and Lehming, 2003). For example, the acetylation (Ac) of histone H3 at lysine 9 and methylation (Me) at lysine 4 is associated with transcriptional activation. To evaluate whether SMAD4 has an effect on histone H3 modification, we performed chromatin immunoprecipitation (ChIP) assay using SMAD4 null cells that had been transfected with either the *Smad4* construct or empty vector. Antibodies specific for Me-K4 and Ac-K9 modified H3 precipitated the hepcidin promoter sequences from extracts of *Smad4* transfected cells and precipitated much weaker bands from extracts of empty vector transfected cells (Figure 4G). The antibody against Me-K4 precipitated significantly more DNA from the hepcidin promoter than the antibody against Ac-K9, suggesting that SMAD4 has a stronger effect on the K4 modification of histone H3. To determine whether treatment with TGF-β or BMP has a similar effect on histone H3K4 methylation, we performed the ChIP assay in Smad4 wild-type cells before and after treatment with TGF-β or BMP. We detected significantly stronger H3K4 methylation of the hepcidin promoter after treatment of either TGF-β or BMP than in un-treated cells (Figure 4H). These data imply that expression of TGF-β/SMAD4 signaling leads to modification of histone H3 in the hepcidin promoter, consistent with the increased expression of endogenous hepcidin (Figure 4D), and increased promoter reporter activity (Figure 4F) in *SMAD4* transfected cells.

Failure of hepcidin induction in SMAD4 deficient liver after administration of IL-6 or iron-dextran

Much is known about the downstream effects of hepcidin on iron homeostasis, but less is known about upstream effectors. IL-6 stimulates hepcidin transcription in cultured primary hepatocytes (Nemeth et al., 2003). It was further demonstrated that the activation of hepcidin transcription by IL-6 does not require HFE or TFR-2 (Lee et al., 2004). To determine if IL-6-dependent induction of hepcidin requires SMAD4, we treated SMAD4 mutant and control mice with IL-6. Our data indicated that hepcidin levels increased to 2.3- and 1.9-fold at 4 and 9 hr after IL-6 administration in wild-type animals (Figure 5A). This level of hepcidin induction is comparable to that found in the primary hepatocytes (Nemeth et al., 2003). In contrast, hepcidin expression in *Smad4$^{Co/Co}$;Alb-Cre* mice maintained very low, from 0.0243 (PBS treated) to 0.03 (4h after IL-6 treatment) and 0.025 (9h after IL-6 treatment), in relation to hepcidin expression in PBS treated control mice, which was set at 1 (Figure 5A). This observation suggests that the absence of SMAD4 not only interfere with baseline hepcidin expression but also blocked the induction of hepcidin by IL-6.

Known as a common mediator for TGF-β superfamily (Heldin et al., 1997; Massague, 1998; ten Dijke and Hill, 2004), SMAD4 is unlikely to be a part of the IL-6 pathway. Based on the data presented earlier suggesting that expression of Smad4 or treatment with its ligands results in an epigenetic modification of histone H3 to a transcriptionally active form, we suspected that rather than mediating IL-6 signaling, Smad4 might be required to open the chromatin of the hepcidin promoter. To determine whether IL-6 signaling pathways are intact in *Smad4$^{Co/Co}$;Alb-Cre* mice, we examined transcription of several acute phase genes known to be induced by IL-6 including CRP, SAA and Albumin. Our data indicated that *Smad4$^{Co/Co}$;Alb-Cre* mice maintained an intact response to the IL-6 treatment, although the responsiveness seemed to be stronger than in the wild-type controls, especially for CRP and SAA-1 (Figures 5B–5D). While these observations revealed a complex relationship between SMAD4 and IL-6, they suggest that failure of IL-6 to induce hepcidin in *Smad4 $^{Co/Co}$;Alb-Cre* mice is not due to a nonspecific block of IL-6 signaling by SMAD4 deficiency.

Hepcidin expression is also induced by iron overload (Pigeon et al., 2001). Because hepcidin inhibits iron absorption, the induction of hepcidin may represent a feedback mechanism, i.e, when iron levels are high, hepcidin expression increase, and the increased hepcidin, in turn, inhibits expression of iron transporters in the intestine to decrease iron absorption. To determine whether SMAD4 plays a role in this process, we treated SMAD4 mutant and control mice with iron-dextran to induce iron overload. Our data indicated that iron overload increased hepcidin expression in control mice by about 2.8-fold 3 days after the treatment. In contrast, hepcidin expression remained low and was nonresponsive to iron treatment in mutant mice (0.03 in PBS-treated and 0.022 in iron-treated mice, Figure 5E). Because *Smad4$^{Co/Co}$;Alb-Cre* mice are already iron overloaded, a potential caveat is that injection of iron-dextran might not result in further iron overload and that the failure of *Smad4$^{Co/Co}$;Alb-Cre* mice to increase hepcidin expression in response to treatment with iron dextran might be due to the lack of change in iron level. To investigate this, we stained liver sections with Prussian blue and found that iron-dextran treatment significantly increased iron levels in both control and mutant livers (Figures 5F–5I). Altogether, these observations indicate that this feedback pathway to induce hepcidin by increased levels of iron cannot operate when SMAD4 is absent.

Discussion

In this study, we investigated the role of the TGF-β/BMP signaling pathway in liver development and maintenance by using *Alb-Cre* to abate SMAD4 expression in the liver specifically.

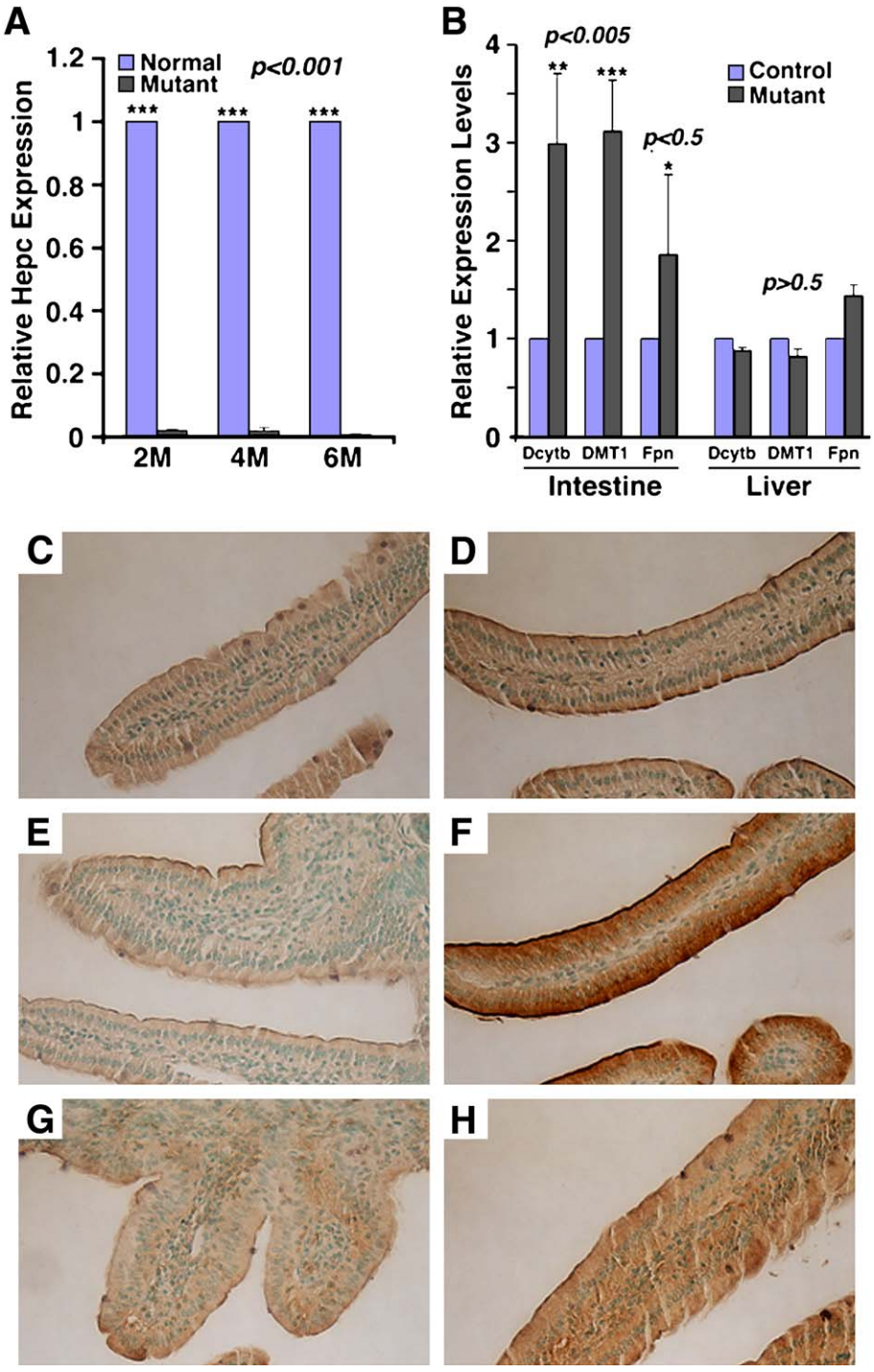

Figure 3. Absence of hepatic SMAD4 results in altered expression of hepcidin and several iron transporters

A) Real-time RT-PCR shows dramatically decreased hepcidin expression in livers of 2-, 4-, and 6-month-old SMAD4 mutant mice compared with normal controls. Hepcidin levels in control mice were arbitrarily assigned a value of 1.

B) Expression of *Dcytb1*, *Dmt1*, and *Fpn* in intestine (duodenum) and liver in 2-month-old *Smad4*$^{Co/Co}$;*Alb-Cre* mice was detected by real-time RT-PCR. Expression levels for each gene in controls were arbitrarily assigned a value of 1.

C–H) Immunohistochemical staining of DCYTB1 (**C** and **D**), DMT1 (**E** and **F**), and FPN (**G** and **H**) in duodenum of control (**C**, **E**, and **G**) and mutant (**D**, **F**, and **H**) mice reveals dramatically increased expression of DCYTB1 and DMT1, and slightly increased expression of FPN.

Values are expressed as mean ± SD.

Unexpectedly, the liver-specific knockout of SMAD4 does not have a major impact on liver development; instead, it results in a dramatic accumulation of iron in the liver of *Smad4*$^{Co/Co}$;*Alb-Cre* mice. In addition, several other organs that have intact SMAD4, including pancreas, kidney, eye and brain, also exhibit accumulation of iron starting from 2 months of age. Thus, our work not only creates a new animal model for hemochromatosis, but also clearly indicates that the liver is a physiological center for regulation of iron homeostasis. The molecular basis for such a phenotype is that liver-specific knockout of SMAD4 in *Smad4*$^{Co/Co}$;*Alb-Cre* mice results in diminished expression of hepcidin, which is made specifically in liver and regulates iron absorption in the duodenum. The absence of hepcidin results in significantly increased expression of iron transporters (FPN1 and DMT1) and ferric reductase (DCYTB1) in small intestine, and enhanced iron absorption, leading to iron overload.

While iron overload was observed in *Smad4* mutant mice of all ages, liver-specific knockout of *Smad4* also caused severe damage of pancreas in older mutant mice. This phenotype may not be explained by the down regulation of hepcidin as

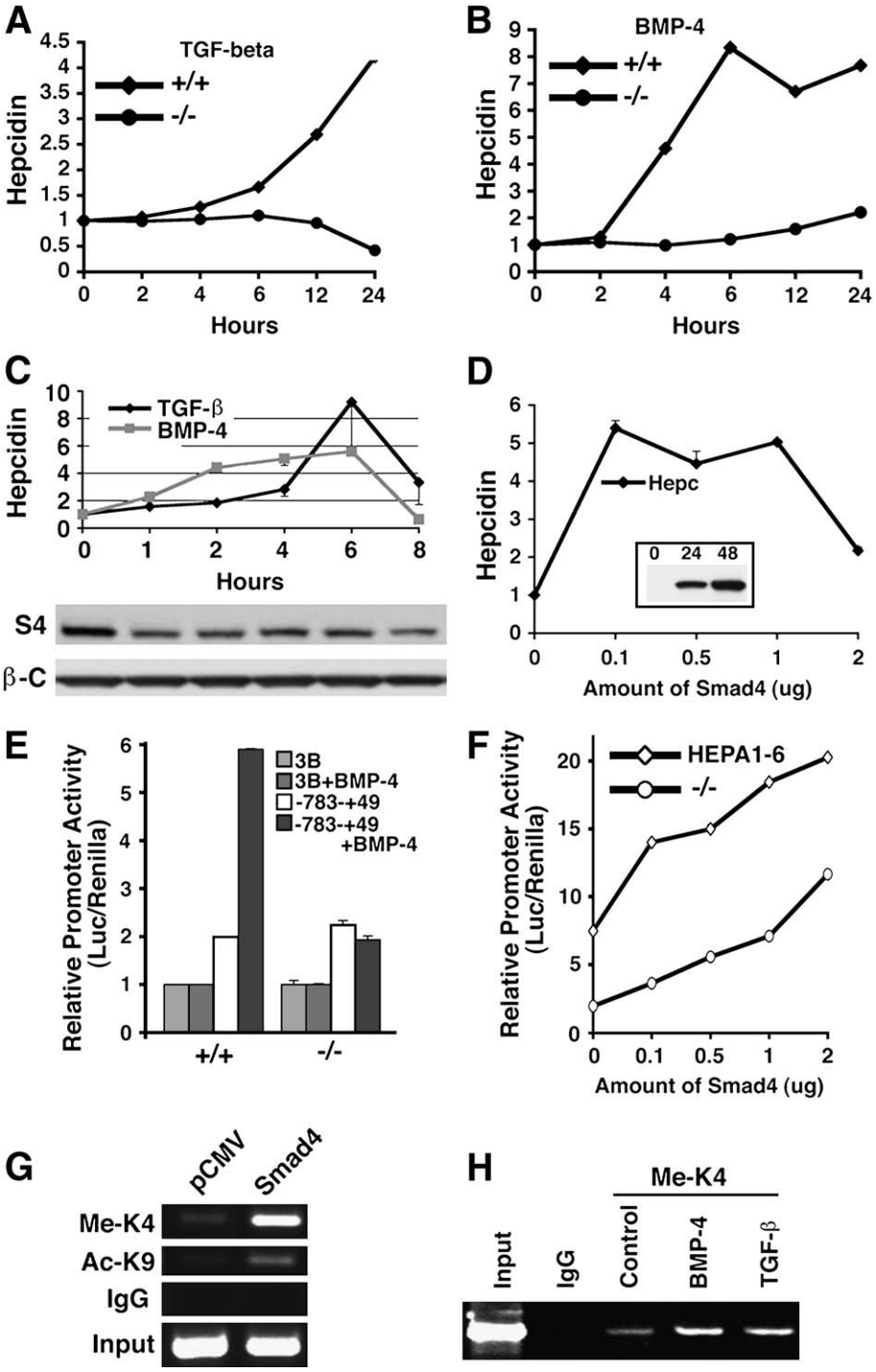

Figure 4. Induction of hepcidin expression by TGF-β signaling requires SMAD4

A and B) TGF-β1 (A) and BMP-4 (B) positively regulate endogenous hepcidin expression through SMAD4 in wild-type SMAD4 (+/+), but not in SMAD4 null (−/−) hepatic cell lines.

C) TGF-β/BMP4 directly induces hepcidin expression. Cells were treated with 10μg/ml cycloheximide for 30 min before TGF- β1 or BMP-4 was added. Data were normalized with cells treated with cycloheximate only. Lower panel shows Smad4 (S4) and β-actin (β-a) levels at the multiple time points up to 8 hr after cycloheximate treatment.

D) Reconstitution of SMAD4 in SMAD4 null cells increased endogenous hepcidin expression. Insert is a Western blot analysis showing SMAD4 levels prior to, 24 hr after, and 48 hr after *Smad4* (1 μg) transfection.

E) Hepcidin promoter activity, as reflected by luciferase assay, is elevated by BMP-4 treatment in wild-type cells but not in the Smad4$^{-/-}$ cells. 3B is a basal vector without hepcidin promoter, which is a fragment containing −783 to +49.

F) Hepcidin promoter activation is SMAD4 dosage dependent in both HEPA1-6 and the MT cells.

G and H) ChIP analysis showing histone H3 acetylation (Ac) at K9 and methylation (Me) at K4 on the hepcidin promoter. The primers for ChIP assay cover −933/−324 fragment of Hepcidin promoter.

Values are expressed as mean ± SD. All assays in (**C**)–(**F**) were performed 48 hr after plasmid transfection.

no such abnormality was reported in mice carrying targeted disruption of hepcidin (Nicolas et al., 2001). Liver produces multiple factors that may be essential for maintenance of pancreatic acinar cells and the production of some of these factors may be impaired in Smad4 mutant mice. To investigate whether levels of factors potentially important for the maintenance of pancreatic acinar cells are altered in Smad4 mutant mice, we have performed microarray analysis on the liver of *Smad4*$^{Co/Co}$*;Alb-Cre* and control mice at 2 and 4 months of age. This study revealed alterations in the expression of many genes other than hepcidin, including several members of the cytochrome p450 family, Igfbp1, Cdkn1a, and Sparc (Table S2). Thus, in addition to iron overload resulting from decreased hepcidin expression, changes in the expression of other genes may contribute to the pathology of Smad4 mutant mice. This possibility remains to be tested in future study.

Importantly, our study reveals a novel role for TGF-β/BMP signaling in induction of hepcidin expression and shows that SMAD4 is required for this activity. Although iron has been considered as a vital metal for the proliferation of all cells inside the body for long time, our experimental data connect iron absorption with growth factor signals. Our data indicated that the

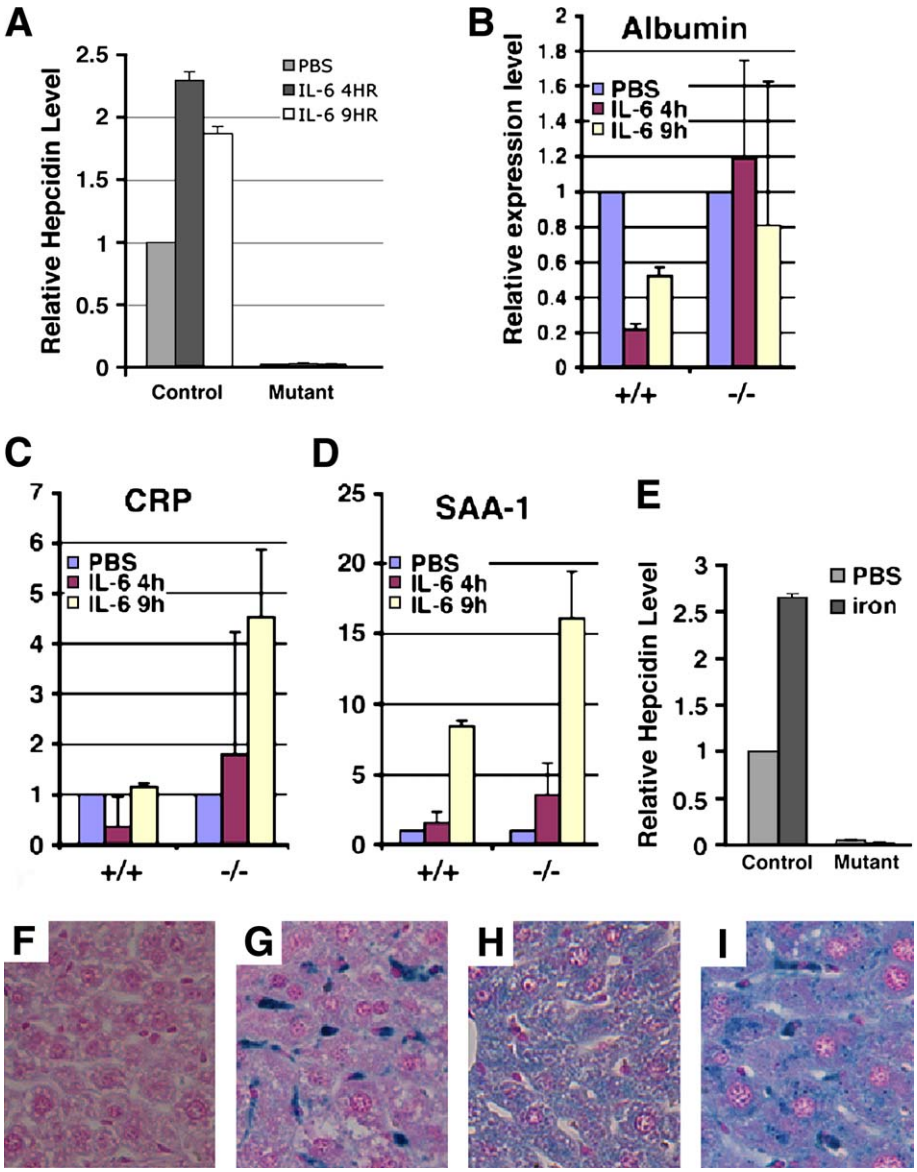

Figure 5. Absence of SMAD4 blocked induction of hepcidin by IL-6 injection and iron overload

A) Hepcidin levels in control and *Smad4*$^{Co/Co}$;*Alb-Cre* mice prior to and after IL-6 injection.

B–D) Expression of IL-6 downstream genes.

E) Hepcidin levels in control and *Smad4*$^{Co/Co}$;*Alb-Cre* mice prior to and after iron-dextran injection.

F–I), Prussian blue staining of control (**F** and **G**) and mutant (**H** and **I**) liver. Of note, the administration of iron-dextran further increased iron levels in mutant liver (**I**) compared with untreated mutant mice (**H**).

Values are expressed as mean ± SD. All animals were 2 months of age, and at least three animals were used for each time point.

increased expression of hepcidin under TGF-β and BMP treatment or SMAD4 overexpression was correlated with increased H3K4 methylation, which is associated with transcriptional activation (He and Lehming, 2003). Therefore, it is possible that TGF-β/SMAD4 signaling is needed for maintaining hepcidin expression by keeping the hepcidin promoter in an active form. Notably, it was shown previously that expression of a dominant-negative mutant TGF-β type II receptor in liver of transgenic mice did not cause obvious abnormalities in several organs examined, including liver, spleen, kidneys, intestine, lung and heart (Kanzler et al., 2001). It also did not cause any decrease in the life span of transgenic mice. We have also examined the liver of Smad3$^{-/-}$ mice (Yang et al., 1999), and found they do not have any increased iron accumulation compared with the control liver (data not shown). These observations suggest that the absence of a single member of the TGF-β subfamily, or loss of a single intracellular mediator for signaling of a subfamily is not sufficient enough to cause iron accumulation. It is possible that BMP signals can maintain normal expression of hepcidin in the absence of TGF-β subfamily signals. Conversely, we also predict that loss of BMP subfamily signaling alone should not have an obvious effect on iron absorption due to the existence of the TGF-β subfamily and/or other subfamilies. Because SMAD4 serves as a common mediator for the TGF-β super family (reviewed in (Heldin et al., 1997; Massague, 1998; ten Dijke and Hill, 2004)), the absence of SMAD4 is predicted to block all the family members that either use SMAD2/SMAD3 (for TGF-β and activin subfamilies), or use SMAD1/SMAD5/ SMAD8 (for BMP subfamily) for signaling, and could therefore lead to a profound effect on iron accumulation that might not occur if only one of the other family members were lost.

In addition to TGF-β signaling, hepcidin expression is also subjected to regulation by a number of factors/conditions, including HFE, c/EBPα, iron, hypoxia, IL-6, and inflammation. The OMIM database divides hemochromatosis into four classes: type1 (HFE mutation related) (Bridle et al., 2003; Gehrke et al., 2003), two juvenile types: type2A (Hemojuvelin related) (Papanikolaou et al., 2004), and type2B (hepcidin related) (Roetto et al., 2003), and type3 (TFR2 related) (Kawabata et al., 2005) all display

dramatic downregulation of hepcidin, indicating hepcidin plays a central role in this disease. We propose that SMAD4 signals play an essential role in maintaining hepcidin expression in liver, as *Smad4$^{Co/Co}$;Alb-Cre* liver contains 50- to 190-fold less hepcidin from 2–6 months of age. Expression levels of FPN1, DMT1, and DCYTB1 increase in intestine of *Smad4$^{Co/Co}$;Alb-Cre* mice, while the expression of HFE, TFR1, and TFR2 does not change. This observation strongly suggests that hepcidin, acts directly on FPN1, DMT1, and DCYTB1 in the intestinal enterocytes, and does not regulate HFE, TFR1, or TFR2 expression in crypt cells.

It was recently demonstrated that hepcidin binds to FPN1. After binding, FPN is internalized and degraded, leading to decreased export of cellular iron (Nemeth et al., 2004b). It was shown that expression of *Dmt1* and *Dcytb1*could be inhibited by iron overload in mice (Ludwiczek et al., 2005). Interestingly, the expression of these genes is increased, rather than decrease in our iron overloaded (*Smad4$^{Co/Co}$;Alb-Cre*) mice. This observation suggests that the diminished level of hepcidin in our mutant mice has overridden the inhibitory effect of iron overload on the expression of *Dmt1* and *Dcytb1*genes. Given the fact that iron overload failed to induce hepcidin expression in our mutant mice (Figure 5E), we suggest that the inhibitory effect of iron overload on expression of *Dmt1* and *Dcytb1*genes is mediated by hepcidin. Although details of the relationship among iron overload, hepcidin, *Dmt1*, *Dcytb1,* and *Fpn* genes remain unclear, our data reveal that diminished hepcidin affects *Dmt1*, *Dcytb1,* and *Fpn* at both the protein and transcript levels. This is an important issue that needs to be addressed in the near future.

Experimental procedures

Mice and cells

Mice carrying the *Smad4* conditional allele (Yang et al., 2002) were crossed with an albumin-Cre transgenic mouse (Yakar et al., 1999). The *Smad4* conditional allele was genotyped as described (Li et al., 2003). HEPA1-6 hepatocyte carcinoma cells, a gift from S.P.S. Monga, were cultured in DMEM with 10% FBS, glutamine and antibiotics. The SMAD4 wild-type (WT) and SMAD4 null (MT) cell lines were derived from *Smad4* wild-type and *Smad4$^{Co/Co}$;Alb-Cre* mouse liver, respectively. These cells have been immortalized spontaneously through serial passing and can be maintained permanently in DMEM, containing 10% FBS, glutamine, antibiotics, and insulin and EGF. Two ng/ml TGF-β1 and 10 ng/ml BMP-4 (R&D) were used to treat the above cells respectively. All the mouse care is in accordance with guidelines of animal user and care committee of NIDDK.

Immunohistochemical staining and Western blot

Immunohistochemical staining using antibodies for DMT1, DCYTB, and FPN were performed as described (LaVaute et al., 2001). SMAD4 antibody for Western blot was purchased from Santa Cruz.

Chromoatin immunoprecipitation

Chromoatin immunoprecipitation (ChIP) assay was performed as described previously (Wang et al., 2004). The antibody against trimethylated Lys4 of histone H3 was purchased from Upstate. The primers used for PCR are Hepc forward 5′-CTG CCA TGT GAA ACC AGT GT-3′ and Hepc reverse 5′-GG AAG CTT ATC ATG CCT TCT GTT CTG CTG-3′ that amplify 609 bp of the hepcidin promoter.

IL-6 treatment and iron overload

Normal and mutant mice at 2 months of age were given IL-6 (PeproTech Inc, Cat. 216-16) through tail vein at 1.5 ug/30 g or equal amount of PBS (for control) for different hours. Iron-dextran (Sigma, Cat. D-8517) was given to normal and mutant mice at 2 months of age by i.p. injection at 20 mg/30 g for 3 days before euthanization.

Prussian blue staining

Slides were stained with K4Fe(CN)6·3H_2O and counterstained with fast red based on a standard procedure.

Measurement of iron and serum transferrin concentrations

Quantification of iron level was performed as described by Torrance and Bothwell (Torrance and Bothwell, 1968). Serum transferrin concentration was measured by Iron/TIBC reagent set (Pointe Scientific Inc., Cat. No. I7504-60).

Cloning hepcidin promoter and luciferase assay

DNA fragment spanning from −783 to +49 of mouse Hepcidin promoter region was cloned into PGL3B vector by Xho1 and HindIII digestion after PCR. Luciferase assays were carried out with Dual-Luciferase System (Promega). Vectors were transfected into the cells by Lipofectamine ^{2000} (Invitrogen).

Realtime RT-PCR and RT-PCR

Total RNA was isolated from the liver of mutant and control mice using RNA-Stat-60 (Tel-Test, Inc.). Quantitative measurement of gene expression was carried out with 7500 Realtime PCR (ABI) equipped with SDS software. β-actin was used as internal control.

Primer information for RT-PCR:

Hepcidin1 F: 5′-CCT ATC TCC ATC AAC AGA TG-3′; Hepcidin1 R: 5′−AAC AGA TAC CAC ACT GGG AA-3′; FPN F: 5′-CCA AGG CAA GAG ATC AAA CCC-3′; FPN R: 5′−CCA CCA GAA ACA CAG ACA CTG C-3′; DMT1 F: 5′-GGT GTT GGA TCC TAA AGA AAA G-3′; DMT1 R: 5′-GAG TAC TCC TCC TCA GGA ATG G-3′; Dcytb F: 5′-CCC ATA CAC GTG TAT TCT GG-3′; Dcytb R: 5′-GGT GAC AAT CCA AAA GAT GAG G-3′; Albumin F: 5′-GAC AAG GAA AGC TGC CTG AC-3′; AlbuminR: 5′-TTC TGC AAA GTC AGC ATT GG-3′; CRP F: 5′-GGG TGG TGC TGA AGT ACG AT-3′; CRP R: 5′-CCA AAG ACT GCT TTG CAT CA-3′; SAA-1 F: 5′-GCG AGC CTA CAC TGA CAT GA-3′; SAA-1 R: 5′-GGC AGT CCA GGA GGT CGT TA-3′.

Following primers were used for RT-PCR:

TFR1 F: 5′-CAA GTA GAT GGA GAT AAC AG-3′; TFR1 R: 5′-CTT CAC ATA GTG TTC ATC TCG-3′; TFR2 F: 5′-CAA GAC CCT CTC AGA CCA TC-3′; TFR2 R: 5′-CAT CTT CAT CGA CCA CCA ACA C-3′; HFE F: 5′-CCC TCT GTG CTT CTC CAC TC-3′; HFE R 5′-GAC ACC ACT CCC AAC TTC GT-3′.

Microarray analysis

RNA isolated from the liver of *Smad4$^{Co/Co}$;Alb-Cre* and wild-type mice at 2 months and 4 months of age was used for microarray based on a procedure described (Tan et al., 2003). Briefly, RNA was labeled and hybridized to microarrays GeneChips from Affymetrix. Each probe usually contained 25 nucleotides and the mouse GeneChips used contained 12,500 genes. Each sample was hybridized to two sets of the same GeneChips and comparisons were made between each pair of samples. Genes showing 2-fold difference in expression at both time points were summarized in the Table S2.

Supplemental data

Supplemental Data include two tables and one figure and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/2/6/399/DC1/.

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